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HISTOCHEMICAL AND MICROCHEMICAL OBSERVATIONS ON THE LIPIDS OF THE INTERSCAPULAR BROWN FAT OF THE FEMALE VESPERTILIONID BAT *MYOTIS LUCIFUGUS LUCIFUGUS*

By

GUY-LAURENT REMILLARD

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Accorded an A. Cressy Morrison Award in Natural Science in 1956 by The New York Academy of Sciences

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HISTOCHEMICAL AND MICROCHEMICAL OBSERVATIONS ON THE LIPIDS OF THE INTERSCAPULAR BROWN FAT OF THE FEMALE VESPERTILIONID BAT *MYOTIS LUCIFUGUS LUCIFUGUS*

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INTRODUCTION

One of the outstanding characteristics of the living organism is its ability to maintain itself in a dynamic equilibrium with its surroundings. The maintenance of the organism is ensured by a series of chemical and physical transformations of energy that, collectively, constitute metabolism.

For the proper functioning of their elaborate metabolic mechanisms, the various animal species have developed different modes of reaction to diversified environmental conditions. In the poikilothermic or cold-blooded animals, the reaction to changes in environmental temperature is such that, as this diminishes, the metabolism and the cell temperature of the animals likewise decrease. Conversely, as the environmental temperature rises, the metabolism and the cell temperature of the poikilothermic animals increase (Benedict, 1932).

Under normal conditions, the reaction of the warm-blooded or homoiothermic animals to changes in environmental temperatures is of a markedly different nature. The homoiotherms are provided with chemical and physical thermoregulatory mechanisms that prevent rapid changes in body temperature and maintain this body temperature usually constant at about 37° C. A fall in the environmental temperature brings into play the *chemical* heat regulation, which immediately calls for an increase in the metabolism of the homoiotherm. By contrast, a rise in the environmental temperature calls for the *physical* heat regulation, which tends to lower the cell temperature of the homoiotherm and results in a decreased metabolism.

However, these chemical and physical thermoregulatory mechanisms prevail as long as the cell temperature of the homoiotherm is not materially altered. When the environmental temperature is decreased markedly, the chemical heat regulation of the homoiotherm deteriorates; the animal no longer can combat the loss of heat due to cold, and its internal temperature falls rapidly. This drop in the cell temperature is accompanied by a lowering of metabolism corresponding, though with markedly different quantitative relations, to the lowering of the metabolism in the poikilotherm (Benedict, 1932; Kayser, 1950). Conversely, with greatly increased environmental temperatures, the physical thermoregulatory mechanisms (consisting in large part of vaporization of water) of the homoiotherm are adversely affected. This results in an increased cell temperature and a rise in metabolism corresponding, though not quantitatively, to the increased metabolism observed in the poikilotherms.

We may therefore conclude that both poikilothermic and homoiothermic animals act qualitatively in the same way. The homoiotherms, however, possess chemical and physical thermoregulatory mechanisms that tend to maintain their body temperature within a narrow range and prevent rapid falls in body temperature in cold environments and rapid increases in body temperature in warm environments. Thus, the body temperature of homoiotherms is maintained within a narrower range than the internal temperature of the poikilotherms.

Between these two main categories comes a third group of animals, the heterotherms, to which belong all the true hibernating mammals. During their hibernating period, the hibernators behave like true poikilotherms: their body temperature reacts slowly to the environmental temperature, but follows its general course (Dontcheff and Kayser, 1935a; Kayser, 1939a, 1940a and 1950; Eisentraut, 1934 and 1940; Benedict and Lee, 1938). However, the heat production of hibernators at different temperatures differs from that of perfect poikilotherms. This difference in behavior has not yet been explained satisfactorily, although Kayser (1950) suspects the influence of additional sources of heat such as muscular movements or hormonal catalysts at certain critical temperatures.

When awakened during the summer months, the hibernating mammals become homoiotherms. They regulate their body temperature, but frequent hypothermias and hyperthermias are noted (Forel, 1887; Mimachi and Weinland, 1911; Simpson, 1911; Groebbels, 1926; Benedict and Lee, 1938; Kayser, 1939a). These hypothermic and hyperthermic conditions have been attributed to very poorly developed physical and chemical thermoregulatory mechanisms (Gelineo, 1938; Kayser, 1939a, 1939b, 1939c, 1949 and 1950; Hiebel and Kayser, 1949). The inability to maintain a homoiothermic state at temperatures lower than thermal neutrality forces the hibernator to enter into winter sleep. Most hibernating mammals can therefore be considered as imperfect homoiotherms during their awakened periods. The only known exception to this general rule is the hibernating bat.

Like all other true hibernators, the bat is poikilothermic during hibernation, but it remains poikilothermic during the awakened period except during its flight at night, when its body temperature and its heat production can be compared with those of the homoiotherms (Merzbacher, 1903 and 1904; Almeida, Fialho, and Couto e Silva, 1926; Burbank and Young, 1934; Eisentraut, 1934 and 1940; Kayser, 1938 and 1950). For this reason, and because bats do not require a prolonged and complex sequence of events to reach the hibernating state, a new metabolic classification of vertebrates has recently been suggested, namely: homoiotherms, heterotherms, bats, and poikilotherms (Hock, 1951).

In addition to the changes in the thermoregulatory mechanisms, various other physiological changes have been studied in hibernating mammals. The literature on hibernation is very extensive, and reference will be made here only to especially valuable reviews on that subject

(Rasmussen, 1916; Johnson, 1931; Ferdmann and Feinschmidt, 1932; Uiberall, 1934; Polimanti, 1912 and 1936; Benedict and Lee, 1938; Emme, 1946; Kayser, 1940c, and 1950). Additional references on various aspects of hibernation may be found in the extensive bibliographies published in these reviews. As pointed out by Benedict and Lee (1938), "the literature on hibernation is more voluminous than accurate.... Most of the earlier literature deals with superficial observations regarding changes in body weight and respiration rate."

Several efforts have been made to contribute some information regarding the factors inducing hibernation. Low temperatures and fasting have long been known to be factors predominant in the production and maintenance of the hibernating state (Kayser, 1940b), but the internal factors responsible for the phenomena of hibernation are still unknown. Numerous attempts to produce artificial hibernation, whether by removal of the various endocrine glands, by narcosis of the nervous system, or by combinations of both experimental procedures have failed thus far to induce prolonged hibernation in winter sleepers (Foster, Foster, and Meyer, 1939; Kayser, 1950). It is surprising to note that, after such repeated failures, a structure that is especially well developed in winter sleepers – the so-called "hibernating gland" of brown adipose tissue – has been so neglected with respect to its possible predominant influence in hibernation. This oversight may be attributed to two main causes:

First, the existence of the "hibernating gland" in certain hibernating species has been denied, while certain nonhibernators possess such a gland (see the lists of some species with or without "hibernating glands" in Rasmussen, 1923, and in Coninx-Giradet, 1927). This fact has therefore produced some doubts as to the gland's importance in hibernation.

Second, the "hibernating gland" bears a close morphological resemblance to the embryonic form of white adipose tissue. Since white fat was long considered as an inert site of fat storage without any specific metabolic activity (Wells, 1940, rightly named his review "Adipose Tissue: A Neglected Subject"), it is not surprising that the brown variety of adipose tissue has been ignored for so long. During the past twenty-five years, however, extensive work has been done on white adipose tissue, and this has recently focused some attention on brown fat, as well.

Brown adipose tissue differs in many of its morphological and physiological properties from adult white adipose tissue. Macroscopically, brown fat has a lobular, glandlike appearance; for this reason, and because it was found first in hibernating animals, it is also known as the "hibernating gland" (Barkow, 1846). Since this gland is especially well developed in the interscapular region of many mammals, it has also been named the "interscapular gland" (Hatai, 1902) or interscapular brown fat.

Although the "interscapular gland" was first related to the hemopoietic organs and then to the ductless glands (Rasmussen, 1923, Coninx-Girardet, 1927), it has long been looked upon as an embryonic form of adipose tissue that acts principally as a reserve for fats and/or as a reticuloendothelial derivative. Despite the structural similarity between brown fat and embryonic white fat, many workers (Hammar, 1895; von Hansemann, 1902; Long, 1922; Rasmussen, 1923; Fawcett, 1952) have shown that brown adipose tissue arises in the embryo from specific *Anlagen* that are constant in their distribution, and no new areas of brown fat appear during postnatal life. A close inspection of the two varieties of adipose tissue reveals distinct differences between their structural components. Brown fat cells are much smaller than those of white fat, and the form and location of the nucleus, as well as the amount of fat and its arrangement within the cell, differ between the two types of tissues.

A most striking difference between the two types of cells is the presence of a large amount of coarsely granular cytoplasm in the brown fat cell that is almost absent in the white; this accounts for the compact appearance of brown fat, in contrast to the open, lacy appearance of white adipose tissue (Rasmussen, 1923). The vascularity of the two varieties also differs. While the arterioles and capillaries in the poorly vascularized white adipose tissue exhibit no particular orientation, the vascular pattern in brown fat resembles that of a lobular gland, and its dense capillary bed can be compared to that of an endocrine gland (Fawcett, 1952).

Moreover, the physiological properties of brown fat also are quite distinct from those of the white. The brown fat of rats shows a much higher respiratory rate and metabolic activity than does white fat (Wertheimer, 1928; Fleischmann, 1929; Ruska and Quast, 1935; Eger and Morgenstern, 1938; Felix and Eger, 1938; Mirski, 1942; Wertheimer and Shapiro, 1948). Furthermore, the respiration of the brown fat of rats in terms of fat-free dry weight is as great as that of the kidney (Hook and Barron, 1941). The rate of phosphorus turnover (Littrell, Martin, and Hartman, 1944) and the uptake of glucose (Adler-Mönnich and Tiberi, 1937) are greater in the brown than in the white fat of rats.

The deposition of glycogen is faster and greater in the brown variety of adipose tissue than in the white. Under certain conditions, the storage of glycogen in brown fat occurs in concentrations that can be compared with those of the liver (Tuerkischer and Wertheimer, 1942; Wertheimer and Shapiro, 1948). Brown fat contains phosphoglucomutase, which is absent from white fat, and it phosphorolyzes glycogen with a markedly higher velocity than does white fat; its phosphorolyzing activity equals that of the liver (Mirski, 1942). Brown adipose tissue also converts the Cori ester into the Robison ester, and it has a great ability to esterify inorganic phosphate on incubation with glycogen in the presence of sodium fluoride (Mirski, 1942). In his extensive experiments, Mirski noted that brown fat is always the most "active" tissue.

Insulin increases the synthesis of glycogen in the brown fat of the normal rat and in the 48-hour-fasted rat during the fasting state as well as after the administration of glucose (Engel and Scott, 1950). Castration or abolition of the thyroid function by thiouracil causes greater deposition of glycogen in the brown fat than in the white fat of rats (Fawcett, 1947 and 1948), while the adrenalectomized rat has a lesser ability to form glycogen in its brown fat after the administration of glucose and insulin than has the normal animal (Engel and Scott, 1950). However, a striking accentuation of the usual stimulating effect of insulin on glycogen accumulation in the brown fat of rats occurs in rats treated with ACTH or cortisone (Engel and Scott, 1950).

While these observations show that brown fat has its own peculiarities with respect to carbohydrate metabolism, another very important group of substances entering into the composition of this tissue, namely the lipids, has received surprisingly little attention.

As with the carbohydrates, the lipids in these two types of adipose tissue exhibit interesting dissimilarities in their response to endocrine deficiencies. Hypophysectomy, as well as adrenalectomy and feeding with thiouracil, causes a marked depletion of the stored lipids in the brown fat of rats, while the white fat of the same animal is much less affected (Fawcett and Jones, 1949). Although no explanation for these observations has been given, the fact that the two varieties of adipose tissue differ markedly in their response to hormones serves to emphasize the possibility that their functions differ significantly.

In an attempt to elucidate the physiological role of the lipids in brown fat, a few workers have analyzed the chemical components of this tissue. The concentration of neutral fats appears to be lower in the brown fat than in the white fat of rats (Clément, 1947a; Fawcett, 1952). Carlier and Evans (1904) have shown that the fats of brown adipose tissue in the hedgehog consist of 90 to 91 per cent olein, 9 to 10 per cent stearin, and traces of palmitin. Conflicting results have been obtained with the determinations of the iodine numbers of the interscapular and subcutaneous adipose tissues: while Shattock (1909) found no significant differences in the iodine numbers in the hedgehog, Coninx-Girardet (1927) reported an iodine number of 71 in the brown fat and of 90 in the white fat of marmots. Fawcett (1952) suggests that the lipid of the brown fat of mice may be more highly saturated than that of the white fat.

Conflicting reports are also found in the literature concerning the other lipid components of interscapular brown fat. While only traces of phospholipids have been noted in the brown fat of hedgehogs (Carlier and Evans, 1904) and rats (Clément, 1947a), various workers found that the brown fat of rats and mice is especially rich in "lipoids" (Cramer, 1920), "a lecithin" (Engelmann, cited in Salmon, 1910, p. 89) or "jecorin, a substance closely related to the lecithins" (Béchamp, cited in Vignes, 1913a). More recently, Tonutti (1936) has observed that the fatty residues from the brown fat of mice is rich in phospholipids, and Fawcett (1952) has demonstrated, by histochemical means, that the brown fat of rats appears richer in phospholipids and acetal phospholipids than the white fat of rats.

According to Cramer (1920), brown fat is rich in "cholesterin" compounds. More recently, Sweet and Hoskins (1940) found one International

Unit of androgen (corresponding to 100 μg. of androsterone) per 50 gm. of tissue in the "hibernating gland" of woodchucks killed during the summer. This quantity of androgen was found by bioassay, on the comb of the capon, to be equal to the concentration of androgen in the testes of the bull. On the other hand, Clément (1947a), using biochemical methods, noted only faint traces of sterols in the interscapular brown fat of rats. The histochemical methods also gave contradictory results on the steroid components of brown adipose tissue. Using the Schultz histochemical test for steroids, Baker, Ingle, and Li (1950) and Fawcett (1952) could not detect any steroid in the brown fat of rats. The birefringence tests for steroids gave negative results in the brown fat of rats studied by Fawcett (1952), while Baker, Ingle, and Li (1950) reported that their birefringence tests indicated the presence of some steroids in the brown fat of rats.

From such scarce and often contradictory observations on the lipid components of brown fat, any speculation on the fat metabolism in this tissue would be highly presumptuous. Nevertheless, for a long time it has been suspected that the lipids play a major role in hibernation. The average respiratory quotient (R.Q.) of about 0.7 in hibernating mammals points to the fact that the lipids play a predominant role in the maintenance of the animal during its period of hibernation. The greater concentration of fatty acids in the liver (Mayer and Schaeffer, 1915), as well as the increased lipase activity of both the liver and the pancreas (Suomalainen, 1935) during hibernation, seems to indicate that the lipid metabolism is increased during this period. Comparisons between the mobilization of fat in ordinary adipose tissue and that occurring in interscapular brown fat have shown that white fat is mobilized in greater concentration (Valentin, 1857, cited in Dubois, 1896; Coninx-Girardet, 1927) and at a faster rate (Beznak and Hasch, 1937; Clément, 1947a, 1947b and 1948) than brown fat.

These experiments show that the metabolism of fat is altered during hibernation, and that the lipids of brown fat are also altered during this period, but they fail to prove that brown adipose tissue plays a predominant role in the production and maintenance of hibernation. Various workers, however, suggest that the "hibernating gland" is more than a specialized form of adipose tissue, and that the brown fat of hibernators plays a definite role in inducing and regulating winter sleep. In support of their views, these authors adduce the following observations:

Injections of brown fat extracts from hibernating hedgehogs were found to reduce the heat production by 20 to 30 per cent in rats and to lower the arterial pressure and the heart rate in rabbits (Wendt, 1937). Similarly, intraperitoneal injections of brown fat extracts from hibernating woodchucks and from thirteen-lined ground squirrels decreased the heat production of white rats (Hook, 1940). Vignes (1913b) states that "hibernectomy" (removal of brown fat) alters the resistance of the "hibernectomized" animal to different drugs; "hibernectomy" in rats and mice increases the resistance of the "hibernectomized" animal to epinephrine and atropine and lowers its resistance to chloroform. The "hibernating gland" apparently modifies enzyme action, for its removal decreased the lipase concentration and the amylolytic power of serum in rats (Vignes, 1913c). From his observations on the antitryptic power and the lipase content of brown fat extracts, Vignes (1913c) concludes that the "hibernating mass" acts as a protein-sparer and favors the utilization of carbohydrates and fats.

Klar's observations (1941) seem to confirm Vignes' hypothesis. By measuring the oxidation-reduction potentials of the interscapular brown fat of hedgehogs, Klar found that these potentials varied throughout the year. They become less positive during hibernation and are even negative in certain cases during the same period of the year. From these results, Klar concluded that brown fat is more active during hibernation, that the dehydrase activity of this tissue becomes predominant during winter sleep, and that his experiments provide additional proof of Vignes' hypothesis.

Chauchard (1944, 1947) greatly emphasizes Klar's observations and maintains that they prove the decisive role of the "hibernating gland" in the maintenance of winter sleep. "It is the activity of the hibernating gland that is responsible, by restraining cellular oxidation, for running the organism at reduced speed, simultaneously diminishing cellular needs and reducing thermogenesis."* (Chauchard, 1944). Chauchard, however, does not contribute any observations of his own to support his dogmatic conclusion. It would seem that much more research must be done before such a conclusion may be justified; and I prefer to conclude, with Kayser, that brown fat plays "an undoubted role in the metabolism of the hibernator, but nothing as yet proves that it decisively intervenes in the 'determinism' of sleep" (Kayser, 1950).

The present investigation was undertaken in the belief that histochemical and microchemical studies on some of the main metabolic constituents of the interscapular brown fat would cast further light on the physiological role of brown adipose tissue in hibernation. The scarce, and mostly contradictory, results obtained with the lipids, which are the most abundant biochemical component of brown fat, suggest the need for their special study. To my knowledge, no one has yet made a systematic study of the lipid composition of brown fat in a normal hibernating mammal throughout the year.

Accordingly, the annual lipid variations, as determined by biochemical and histochemical means, have been followed in the "hibernating gland" of the vespertilionid bat *Myotis lucifugus lucifugus*. This species was chosen for a number of reasons; much is already known about its life history and habits, specimens may be obtained in adequate numbers throughout the year, and the species is easily maintained in artificial hibernation in the laboratory.

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*Editor's translation.

The results obtained in this study will be discussed in light of the pertinent data in the literature, and tentative interpretations of these results will be given.

MATERIALS AND METHODS

The Experimental Animals

The bats used for the present work were collected during a period extending from May 1952 to April 1953. The three groups of summer specimens were taken from the attics of old houses near Ithaca, N.Y. One of these groups was collected on May 23, 1952, the second on August 8, 1952, and the third on August 29 of the same year. The winter specimens were collected from Woodward Cave, Centre County, Pa.; a series of these specimens was collected on December 8, 1952, while the other series was collected on March 21, 1953. All of the bats used in the experiments were females. Adult female bats were chosen because of the difficulty of collecting adult males throughout the summer months.

All the animals collected during the summer months were kept in wire cages at room temperature for a period of 23 to 28 hours and were given water, but no food. At the end of this period, they were killed, and autopsies were performed, as described below.

The specimens collected during hibernation were brought back to the laboratory in Ithaca within the shortest possible time (about 10 hours) and placed immediately in a room where a temperature of about 10° C. was consistently maintained. These hibernating bats were left in the refrigerated room with water only, for a variable period of time (30 to 40 days), after which they were brought to room temperature and killed. All of the animals were killed by placing them in a closed chamber filled with illuminating gas. Death occurred within the next 10 to 15 minutes, and the interscapular brown fat was removed immediately; the time for the entire process never exceeded 25 minutes.

The excised interscapular brown fat was either transferred to a proper fixative for histochemical analysis or placed in a clean, dry watch glass for weighing. Weighing was performed in the shortest possible time, and the weighed brown-fat tissues were immediately transferred to a glass-stoppered Erlenmeyer flask containing 15 ml. of cold acetone. Those tissues were used for microchemical determinations.

Histological and Histochemical Methods

For routine morphological studies, the interscapular brown adipose tissue was fixed in Helly's, Rossman's, or Bouin's fluid, embedded in

Tissuemat,* and sectioned at 2, 4, and 6 μ. The sections were stained with hematoxylin and eosin, by Masson's trichrome method, and by Van Gieson's fast green method (Lillie, 1948).

Tissues fixed in Helly's fluid and postchromed in 3 per cent potassium dichromate for 48 hours at 37° C. were embedded in Tissuemat and sectioned at five μ. These sections were stained for mitochondria by means of Heidenhain's iron alum hematoxylin (Lillie, 1948) and Mallory's phosphotungstic acid iron hematoxylin (Mallory, 1938). Mitochondria were also stained with Cain's modification of the Altmann aniline acid fuchsin technique (Cain, 1948a), and counterstained with toluidine blue.

Brown fat tissues fixed in Rossman's fluid were embedded in Tissuemat and sectioned at five μ. The sections were stained for reticulum by the McManus periodic acid-Schiff method (Lillie, 1948). Reticulum also was demonstrated in Helly-fixed tissues by Gomori's silver oxide solution (Lillie, 1948).

For the study of lipids, the tissues were fixed in 10 per cent neutral formalin, formal-saline or formal-calcium, treated with potassium dichromate and embedded in gelatin (Baker, 1944). Sections were cut on the freezing microtome at 5 to 10 μ and stained by Baker's (1949) revised Sudan black method, using Mayer's carmalum as a counterstain.

To distinguish between "neutral" and "acidic" lipids, the Lorrain Smith Nile blue sulfate reaction (Smith, 1907; Baker, 1944; Cain, 1947a and 1950; Lison, 1953) was used. The tissues were fixed in formal-calcium, treated with potassium dichromate, embedded in gelatin (Baker, 1944), and cut at 5 to 10 μ on the freezing microtome. Some sections were stained with a 1 per cent Nile blue sulfate solution, while other sections were stained with a 0.02 per cent Nile blue sulfate solution, as recommended by Cain (1947a). A comparison between the sections stained in the 1 per cent solution and those stained in the 0.02 per cent solution should indicate, according to Cain (1947a), the location of fatty acids if other fatty acids besides oleic acid are present in the tissue.

Phospholipids were stained with acid-hematein by Baker's modification of the Smith-Dietrich reaction (Baker, 1946 and 1947; Cain, 1947b). Tissues fixed in formal-calcium-cadmium were carefully treated as directed by Baker (1946), and the specificity of this test was checked by the pyridine extraction† test (Baker, 1946).

Tissues fixed in 10 per cent neutral formalin or in formal-calcium were examined for steroids. Unstained frozen sections (with or without previous embedding in gelatin) were mounted in Farrant's medium or extracted during a four-hour period in acetone or in ethanol before being mounted. These sections were examined with the polarizing microscope for the presence of anisotropic lipids.

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*Tissuemat is a paraffinlike substance that has physical properties which make it superior to paraffin for certain purposes. It is a product of the Fisher Scientific Co., New York, N.Y.

†Throughout this article, the word "extraction" is used in its special reference to a procedure in which a piece of tissue is placed in a heated solvent to ensure the removal of certain chemical substances that resist the usual histological treatments.

Digitonin in alcoholic solution (Brunswik, 1922; Lison, 1953) or in warm (37° C.) aqueous solution (Leulier and Noel, 1926; Leulier and Revol, 1930; Bennett, 1940) was applied to frozen sections of tissues fixed in formal-calcium or in 10 per cent neutral formalin, embedded in gelatin, and sectioned at 5 to 10 μ. These sections were examined between Nicol prisms for acetone-insoluble digitonides indicative of the presence of 3β-hydroxy-sterols (Dempsey and Bassett, 1943; Cain, 1950).

The histochemical color tests for steroids were applied to frozen sections of tissues fixed in 10 per cent neutral formalin or in formal-calcium, embedded in gelatin, and sectioned at 5 to 10 μ on the freezing microtome. The Romieu method (Lison, 1953) and the Schultz I method (Schultz and Lohr, 1925; Lison, 1953) are modifications of the Libermann-Burchardt test and involve the oxidation of cholesterol by acetic anhydride. The Schultz II method (Schultz and Lohr, 1925; Everett, 1945; Lison, 1953) are modification of the Lifschutz "oxycholesterol" test in which cholesterol is first oxidized with either ferric salts or atmospheric oxygen in the presence of sunlight (Cain, 1950).

Steroids were also investigated by means of the original Schultz test (Schultz, 1924) in which cholesterol is not treated by any oxidizing agent. In this test, glacial acetic acid is employed together with concentrated sulfuric acid, and a positive reaction is given by the oxidation products of steroids (Cain, 1950).

Microchemical Methods

The interscapular brown fat tissues that had been stored in 15 ml. of cold acetone were transferred into Potter-Elvehjem homogenizer tubes and homogenized in cold acetone. A total of nine homogenized mixtures were used for the biochemical determinations. The list of these homogenates is recorded in TABLE 1, together with the total number and the fresh weight of the interscapular lobes included in each homogenate. The first eight groups of lobes were homogenized within 24 hours after the animals had been collected. The ninth group of lobes was taken from animals collected on March 21 and kept in artificial hibernation until April 21. On this last date, the lobes were removed from the animals, and these lobes were homogenized 24 hours later.

Each homogenized mixture, together with the cold acetone washings, was transferred to a 50 ml. glass-stoppered volumetric flask, and the volume of each mixture was adjusted to 50 ml. with acetone. Aliquots were transferred to tared porcelain crucibles or to tared porcelain combustion boats for the determination of water and total solids in the tissues, as described below. The rest of the homogenized mixture was filtered into a suction flask through a glass-sintered filter tube of fine porosity. The filtrate and the acetone washings were transferred into glass-stoppered Erlenmeyer flasks and used for the various determinations of lipids in the acetone fractions, while the precipitate was extracted with at least three 10-ml. portions of ethanol-ether (3:1) and filtered in the same manner as the remaining homogenized mixture. The extraction of the precipitate was repeated with at least two 10-ml. portions of ethanol, followed by at least two 10-ml. portions of ether. The combined ethanol-ether filtrates and the ethanol-ether washings were transferred into a glass-stoppered volumetric flask and stored in the refrigerator. These combined ethanol-ether filtrates and washings were subsequently used for the determinations of lipids in the ethanol-ether fractions of brown fat.

TABLE 1

Number of group	*Date of collection of group of animals*	*Total number of interscapular lobes removed and used for biochemical analysis*	*Total weight of the interscapular lobes (in mg.)*	*Average weight of one interscapular lobe (in mg.)*
1	May 23, 1952	10	304.5	30.45
2	May 23, 1952	10	342.9	34.29
3	August 8, 1952	10	127.1	12.71
4	August 8, 1952	10	137.6	13.76
5	August 29, 1952	6	492.1	82.02
6	August 29, 1952	2	160.4	80.20
7	December 8, 1952	10	481.6	48.16
8	December 8, 1952	10	432.0	43.20
9	March 21, 1953	3	109.1	36.37

Determination of water and total solids. Two methods were used for the determinations of water and total solids in the brown fat extracts. These methods will be described as Method No. 1 and Method No. 2:

Method No. 1. Four-ml. aliquots of the homogenized mixture of tissues in acetone were carefully measured into tared porcelain crucibles and evaporated in an oven at 60° C. for 24 hours. The crucibles containing the dry residues were next cooled to room temperature in a desiccator over $CaCl_2$ and weighed at room temperature. The evaporation procedure at 60° C. was repeated until a constant weight was obtained.

Method No. 2. One-ml. aliquots of the homogenized mixture of tissues in acetone were carefully measured into tared combustion boats (Coors porcelain, size No. 4) and placed in the inner horizontal jacket of an Abderhalden vacuum drying (Pyrex interjoint) apparatus*. The evaporation under vacuum was made at a constant temperature by using acetone

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*The Scientific Glass Apparatus Company, Bloomfield, N.J.

(placed in a 100-ml. round-bottomed distilling flask) as a reflux mixture. P_2O_5 was used as the dehydrating agent, and glass wool was placed in the receiving flask to increase the absorbing area and also to prevent a contact between the P_2O_5 and the water from the vacuum pump. Suction flasks were also placed between the vacuum pump and the Abderhalden apparatus to act as additional safety valves, while glass beads were added to the acetone to prevent bumping.

After a 2-hour desiccation, the combustion boats containing the dry residues were removed from the apparatus and allowed to cool to room temperature in a desiccator over $CaCl_2$. The combustion boats plus the dry residues were next weighed at room temperature. The desiccating procedure in the Abderhalden apparatus was repeated until a constant weight was obtained. In most cases, it was necessary to repeat the desiccating procedure only once with this method, while a total of at least 4 desiccations was required to obtain a constant weight with Method No. 1.

Determination of total lipids. The total lipids (total fatty acids plus total cholesterol) of the interscapular brown fat extracts were determined by Bloor's method for the determination of small amounts of lipids in blood plasma (Bloor, 1928 and 1947). Since it was necessary to adapt this method for the determination of lipids in brown fat extracts, slight modifications were introduced. The procedure used was as follows:

Twenty-ml. portions of the extracts were measured into 50 ml. Erlenmeyer flasks. An equal volume of solvent alone was placed into another Erlenmeyer flask and carried all the way through the analytical procedure as a blank.

Saponification was accomplished by adding 2 ml. of a fresh solution of sodium ethylate (prepared exactly as recommended by Bloor, 1928) to each flask and evaporating on the steam bath to almost complete dryness. The last traces of solvent vapor were swept out of the flask by a very gentle stream of air, leaving a pasty residue. This residue was acidified with 1 ml. of dilute sulfuric acid (1 H_2SO_4 conc.:3 H_2O). The acidified material was heated on the steam bath for 1 min. To the hot mixture were then added at least 3 portions (usually one 10-ml. portion followed by two 5-ml. portions) of a petroleum ether-chloroform mixture (7 parts of petroleum ether, with a boiling point (b.p.) of 30 to 60° C. to 1 part of $CHCl_3$), which was thereby made to boil on the steam bath. The flask was then rotated gently at the boiling temperature on the steam bath for 3 to 4 min., after which the solvent was completely poured off from the watery residue into a 25-ml. glass-stoppered volumetric flask. After the extractions were completed, the volumetric flasks containing the petroleum ether-chloroform extracts were brought to room temperature, made up to volume with petroleum ether-chloroform, the contents well mixed, and the flasks stoppered tightly.

For the oxidation procedure, 5-ml. aliquots of the petroleum ether-chloroform extracts were transferred to 50-ml. glass-stoppered Erlenmeyer flasks and treated according to Bloor's colorimetric procedure for the determination of total lipids in blood plasma (Bloor, 1947). This procedure was followed very closely, and all glassware used in the oxidative determinations was cleaned with detergent, rinsed, soaked in a dichromate-H_2SO_4 mixture for at least 60 minutes in an oven at 126°(± 2°) C., rinsed at least 3 times with glass-distilled water, dried with heat, and tightly stoppered until used.

For the determination of the optical transmittance, the contents were placed in selected 19 × 105 mm. round cuvettes No. 14-309* and read against the blanks set at 100 per cent transmittance of a Coleman No. 14 Universal Spectrophotometer at a wave length of 600 mμ with a PC-4 filter.

The standard curve was prepared using a standard solution of 0.1 mg. of purified stearic acid per ml. of petroleum ether-chloroform (7:1). Appropriate aliquots were carried through the entire procedure, and the resulting readings were plotted on semilogarithmic paper (FIGURE 1). The values for total lipids (total fatty acids plus total cholesterol) of the samples of brown fat were determined from this standard curve.

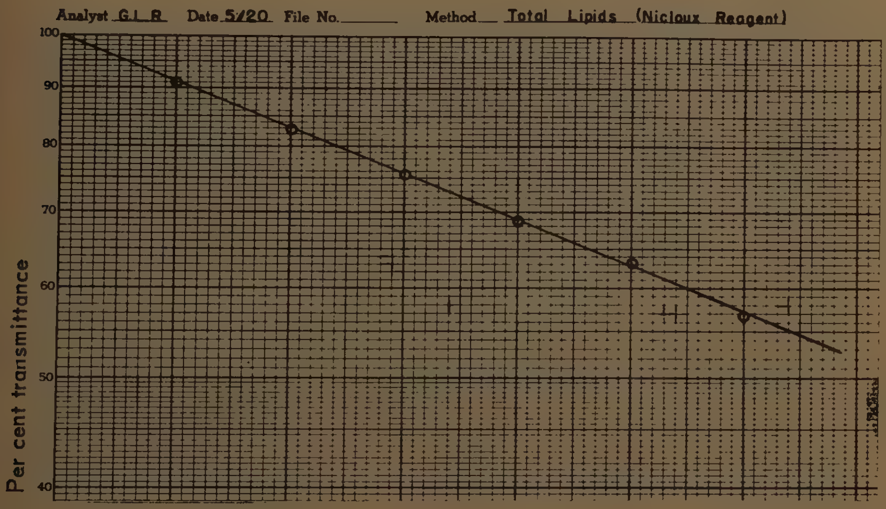

FIGURE 1. Determination of the optical transmittance of the total lipids in the extracts of interscapular brown fat by a modification of Bloor's method (Bloor, 1947). The concentration of the standard solution is 0.1 mg. of stearic acid per ml of $CHCl_3$, a petroleum ether (7:1 solution).

The readings were made on a Coleman No. 14 Universal spectrophotometer; cell length is 19 × 105 mm., the wave length is 600 mμ.

Determination of total cholesterol. For the determination of total cholesterol in the extracts from the interscapular brown fat, the method of Abell and his collaborators (Abell, Levy, Brodie, and Kendall, 1952) for the estimation of total cholesterol in serum was used. The reagents were prepared according to the specifications recommended by these workers, and their analytical procedure was followed very closely. The method involved (1) treatment of a 1-ml. sample of the tissue extract of tissues with alcoholic KOH to liberate the cholesterol from the lipoprotein complexes and to saponify the cholesterol esters; (2) extraction of the cholesterol into a measured (10-ml.) volume of petroleum ether (b.p. 68° C., redistilled) after dilution of the alcoholic solution with 5 ml. of glass-distilled water; and (3) measurement of the cholesterol in 4-ml. aliquots of the petroleum ether layer by means of the Liebermann-Burchard color reaction.

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*Coleman Instruments Incorporated, New York, N.Y.

The optical transmittance of each sample was read against 2 blanks (prepared according to the procedure described by Abell, Levy, Brodie, and Kendall, 1952) in a Coleman No. 14 Universal Spectrophotometer at a wave length of 620 $m\mu$ with a PC-4 filter and selected 19 × 105 mm. round cuvettes.

The standard cholesterol solution was prepared as follows: 100 grams of chemically pure cholesterol* were recrystallized 4 times from redistilled absolute alcohol and brought to volume with redistilled absolute alcohol in a 250-ml. glass-stoppered volumetric flask. In this manner we obtained a standard alcoholic solution of purified cholesterol that contained 0.4 mg. of purified cholesterol per ml. of redistilled absolute alcohol. The values for the standards and the samples of brown fat were calculated from the optical densities as recommended by Abell, Levy, Brodie, and Kendall (1952).

Determination of total phospholipids. The total lipid phosphorus content of the extracts of interscapular brown fat was analyzed according to the revised method of Sumner (Sumner, 1944; Sumner and Somers, 1949; Tsuji, 1950). This analytical procedure was followed very closely, and the optical transmittance was read at a wave length of 700 $m\mu$ in a Coleman No. 14 Universal Spectrophotometer with selected 19 × 105 mm. round cuvettes and a PC-5 filter, as directed in Tsuji's modifications of Sumner's method.

The total phospholipid content of the tissues can be obtained from the determinations of the total lipid phosphorus, since the "average" phospholipid contains 4 per cent phosphorus.

OBSERVATIONS

Anatomical distribution of the interscapular brown fat. The bulk of the brown fat in the hibernating adult *Myotis lucifugus lucifugus* lies immediately under the skin in the interscapular fossa. It is somewhat butterfly-shaped in dorsal aspect, and it appears to be a single mass of tissue.

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*Fisher Scientific Company, New York, N.Y.

However, when the thin layer of overlying connective tissue and white fat has been removed, the apparently single mass can be separated easily into two symmetrical, triangular lobes on either side of the mid-dorsal line, in the depression between the scapulae (FIGURES 2 and 3). The two lobes lie superficially to the muscles, and their lateral and ventral outlines are slightly molded by these muscles. Thin strands of connective tissue secure each lobe to the underlying structures. The two lobes are referred to jointly as the "interscapular gland" or "hibernating gland."

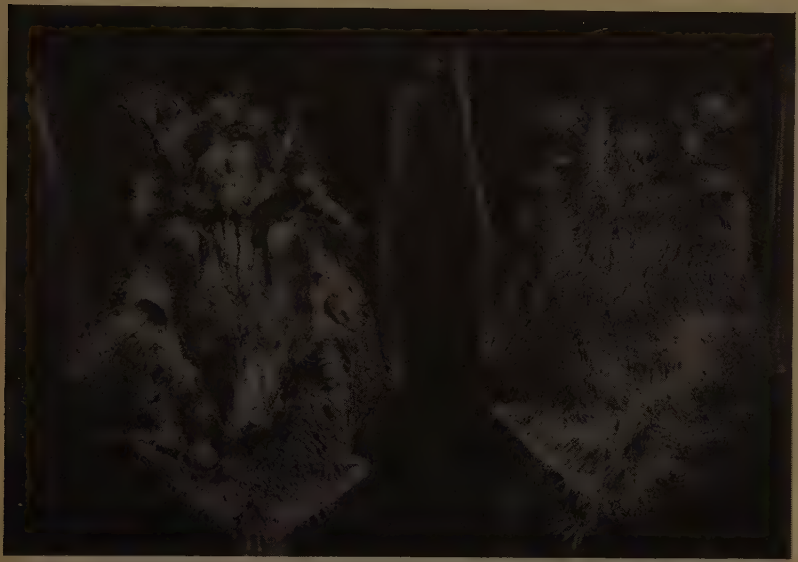

FIGURE 2. Dorsal view of two female vespertilionid bats, *Myotis lucifugus lucifugus*, collected during the first week of August. The life-size specimen on the right has been left intact. On the specimen to the left, the skin and superficial fascia of the thoracic and lumbar regions of the back, together with the skin and superficial fascia of the cervical and occipital regions, have been excised. The dense connective tissue and the scanty amount of white fat in close contact with the masses of brown fat have been removed. The two lobes of brown fat can be seen on either side in the midline of the interscapular fossa. The relatively small size of these lobes and their very light color can be observed.

Each lobe of the "gland" extends posteriorly to the inferior angle of the scapula while, anteriorly, each lobe overlaps the occipital region of the head. Three borders are distinguishable in each lobe: the lateral border extends to the vertebral border of the scapula; the medial border is broadly in contact with the medial border of the other lobe; and the cephalic or anterior border is intimately associated with relatively large blood vessels and nerves supplying the brown fat masses as well as the muscles of the back and neck. Thin lateral arms of brown fat extend from the lateral angles of each triangular lobe and curve around the sides of the neck. These lateral arms of brown adipose tissue surround two relatively large blood vessels and one nerve bundle that penetrate the interscapular masses of brown fat and also supply the muscles of the thoracic and lower cervical region. This nerve is derived possibly, from the lower cervical and upper thoracic nerves, as in the hedgehog (Carlier, 1893).

In adult bats during the summer months, and in young bats, the two interscapular lobes are clearly separated from each other, and each lobe fills a smaller part of the interscapular fossa (FIGURE 1). The color of

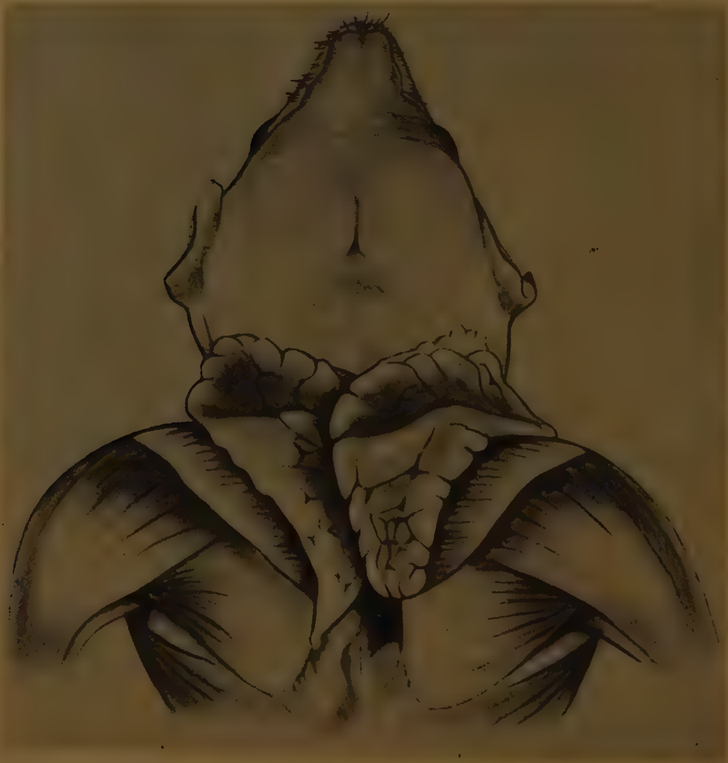

FIGURE 3. Dorsal view of the thorax, shoulders, neck, and head of a vespertilionid bat, *Myotis lucifugus lucifugus*, collected during hibernation. The skin and superficial fascia have been removed entirely. The dense connective tissue and white fat in close contact with the surface of the interscapular lobes of brown adipose tissue also have been carefully excised. The interscapular lobes are seen to be much larger than those shown in FIGURE 2. The lobe on the right is seen to overlap that on the left, and both lobes project cephalad to cover a part of the occipital region of the head. Camera lucida drawing.

the interscapular masses also varies. The interscapular lobes are salmon colored during hibernation and become a deep red during the awakened period. These variations in color are mostly due to the changes in vascularity of brown fat as well as to the variable amounts of white fat that overlie the lobes and that are incorporated into the capsule of these lobes.

Microscopic organization of the interscapular brown fat. Both lobes of interscapular brown fat are identical in histological appearance. Each lobe is covered by a thin capsule of fibrous connective tissue in which relatively large blood vessels, nerves, and a variable amount of white fat cells are found (FIGURE 4). From the capsule, septa of loose areolar connective tissue penetrate the lobe and divide it into irregular, well-defined units or lobules (FIGURE 5). Blood vessels, nerve fibers and, in places, a few white fat cells can be seen in the septa (FIGURES 5 and 6).

Branches from the interlobular arteries enter the lobules (FIGURES 5 and 7), and the dichotomous branching of an intralobular artery produces

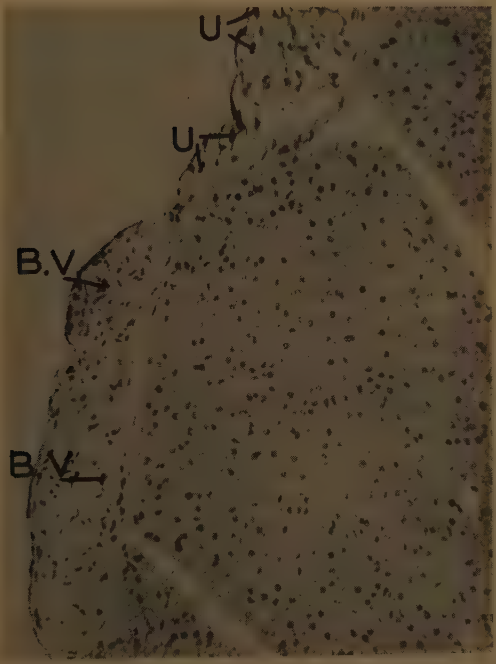

FIGURE 4. Margin of an interscapular lobe of brown fat, showing a part of the connective-tissue capsule with its nerves, blood vessels, and white (unilocular) adipose cells. The looser areolar connective tissue can be seen to form septa that divide the lobe into definite lobules of brown adipose tissue. Section stained by Orth's fast green method.

Symbols: *U* = unilocular adipose cell; *N* = nerve fibers; *B.V.* = blood vessels. × 150.

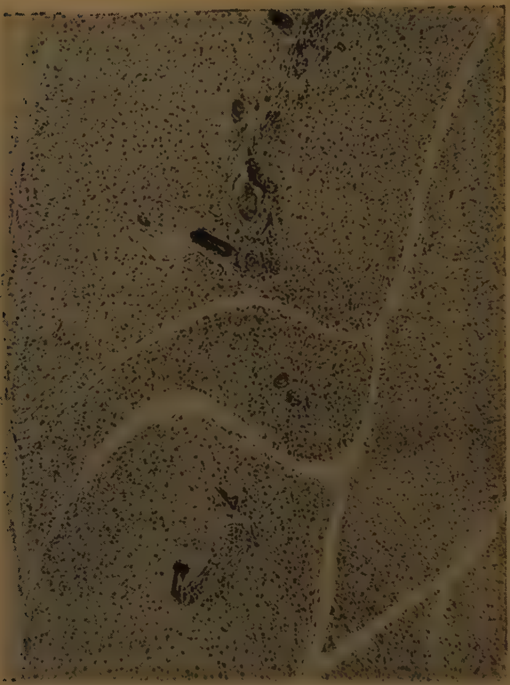

FIGURE 5. Section of an interscapular lobe of brown fat stained by Masson's trichrome procedure. The loose areolar connective-tissue septa divide the lobe into irregular, well-defined lobules. The intralobular septa have an abundant vascular supply. × 50.

a very rich capillary network that seems to surround each cell of the brown fat (FIGURES 8, 9, and 10). Thus, the morphological organization and the vascular pattern of the interscapular brown fat resemble that of a lobular gland, and the dense arrangement of the capillary bed may be compared with that of the endocrine glands.

The parenchyma of a lobule is composed of densely packed epithelioid polygonal cells (FIGURES 10 and 11). A few white fat cells occasionally can be seen in the lobular parenchyma. Each polygonal brown fat cell is clothed with interlacing reticular fibers of varying thickness and random orientation (FIGURES 12 and 13). Through the meshes of the network, the blood capillaries run a tortuous course to become intimately associated with individual brown fat cells (FIGURE 13).

In sections, brown fat cells are seen to vary in size at different periods of the year. The diameter of a cell is about 12 to 15 μ in tissues collected during the summer months. At the end of August (approximately one month before the onset of hibernation), the size of a brown fat cell has increased slightly from 15 to 18 μ. During the hibernating period, there

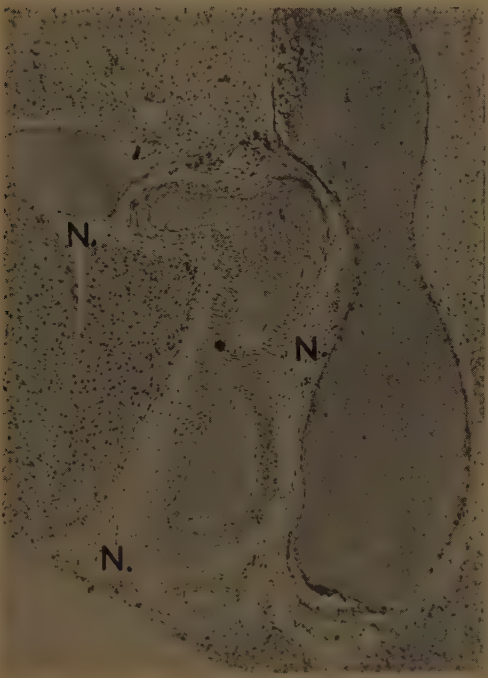

FIGURE 6. Large septum near the periphery of an interscapular lobe of brown fat at the point of entrance of the main blood vessels that supply the masses of brown fat. Section stained by Orth's fast green method. × 50.
Symbol: *N* = nerve fibers.

seems to be a decrease in the size of each brown adipose cell. Consequently, the diameter of a cell is about 12 to 18μ in tissues collected in December, while it is 11 to 16μ in tissues collected in March. Studies of the diameters of the brown fat cells in the interscapular lobes of *Myotis lucifugus lucifugus* therefore suggest that these cells increase gradually in size at the approach of the hibernating period and subsequently undergo a gradual decrease in size during the hibernating period.

The nucleus of a brown fat cell is nearly spherical in shape, with a diameter of about 5 to 6μ. The nucleus is either central or slightly eccentric in position, but it is rarely, if ever, flattened against the cell membrane. The chromatin material forms a dense granular network (FIGURES 8, 9, and 10), in the meshes of which a distinct nucleolus sometimes can be seen.

The cytoplasm of a brown fat cell is rather abundant and coarsely granular, and it is laden with lipid droplets. Because of the presence of these numerous droplets in its cytoplasm, brown adipose tissue is often called "multilocular" adipose tissue, in contrast to the designation of "unilocular" adipose tissue for the white variety. The lipid droplets of brown fat cells have a uniform spherical shape, but their number and size vary with the time of the year.

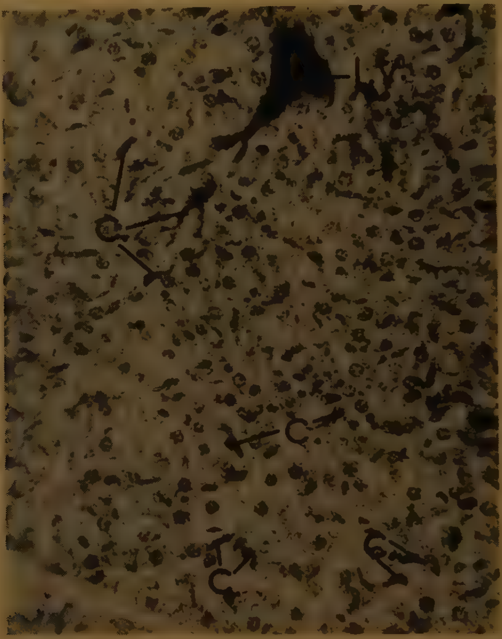

FIGURE 7. Frozen section of an interscapular lobe stained by Baker's acid-hematein method after pyridine extraction. This procedure removes the lipids, but intensifies the staining of certain proteins. With this method, the nuclei of the brown adipose cells are conspicuous. The dichotomous branching of the large intralobular blood vessel in the upper part of the figure also is demonstrated. The blood capillaries appear as black streaks. × 240.

Symbols: *I.V.* = intralobular blood vessel; *C* = blood capillary.

Since publication of the relatively old work of Rasmussen (1923) on the structural changes in the brown fat tissues of the American marmot *Marmota monax rufescens*, surprisingly few morphological studies on the brown fat of hibernating mammals have been made. In the light of recent progress in the field of histochemistry, I have used the histochemical approach to study the qualitative variations in the structure and the chemical composition of the cytoplasmic components of brown fat at different periods of the year in the female bat *Myotis lucifugus lucifugus*. Quantitative microchemical determinations of some of the lipid components of brown fat in the same animal also were made in the present investigation. The histochemical observations will be presented first, and the histochemistry of the lipids in the cytoplasm of brown fat cells will be reported separately for each of the substances and structures studied.

Structural variations in the lipid droplets or "fat vacuoles" of the brown fat cell. Examination of the entire interscapular lobe of brown fat reveals a different picture at various times of the year. These changes in the picture of a lobe are especially conspicuous in sections treated with Sudan black B.

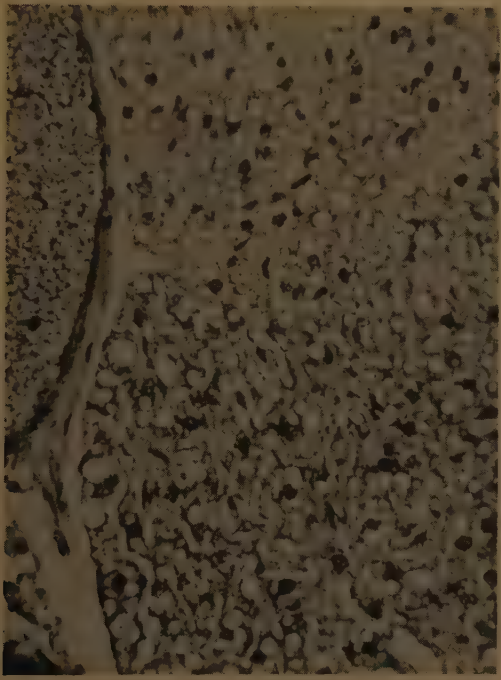

FIGURE 8. Section of a lobe of interscapular brown fat stained by Orth's fast green method. The numerous capillaries appear as thick white lines within the lobules. The variable sizes of the round cytoplasmic vacuoles in the multilocular cells of brown fat also are demonstrated. × 240.

A uniform picture is seen throughout the whole extent of the interscapular lobe in frozen sections cut from the interscapular brown fat tissues collected in December. Each cell is heavily laden with fat droplets of uniform size, and no particular area of denser coloration can be distinguished in sections treated with Sudan black B (FIGURES 14 and 15). However, in tissues collected at the end of the hibernating period, a relatively narrow "cortex" can be distinguished from a wide "medulla" in each interscapular lobe (FIGURE 16). The brown fat cells of the "cortex" are densely packed with lipid droplets that are colored intensely with Sudan black B, while the cells of the "medulla" are less heavily filled with lipid droplets of a lighter coloration. The cortical region of the lobe at the end of the hibernating period (FIGURE 17) closely resembles the entire lobe of brown fat collected in December, but the medullary region of the interscapular lobe at the end of the hibernating period has a configuration of its own. The number of lipid droplets and the intensity of coloration of these droplets have distinctly decreased within a medullary cell, but their size and distribution seem unaltered.

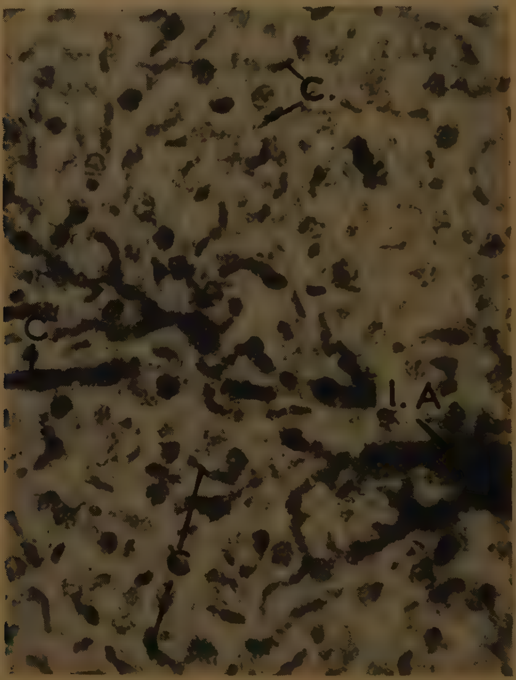

FIGURE 9. The dichotomous branching of the large intralobular artery at the right of this figure can be seen in this frozen section that has been treated by Baker's acid-hematein after pyridine extraction. The blood capillaries appear as thick black lines around individual cells of brown fat. × 450.

Symbols: *I.A.* = intralobular artery; *C* = blood capillary.

In the interscapular lobes collected from May to the end of August, a very narrow cortical region and a medullary region are still recognizable (FIGURES 18 and 19), but various distinctive features characterize the lobes collected during this period of the year. The first striking feature of brown fat tissues collected during these summer months is the presence of very large (12 to 16 μ) lipid droplets in some of the brown fat cells (FIGURES 8, 9, 20, and 21). These very large lipid droplets can be found in cells anywhere within the lobules, but the greatest abundance of them seems to occur in cells in close vicinity to the walls of the larger blood vessels and at the periphery of the lobe (FIGURES 18-21). Their size and their general appearance at first suggest that they are droplets of white fat cells. However, a closer examination of these apparently unilocular cells in thin frozen sections colored with Sudan black B reveals the presence of additional tiny spherical droplets within each of these apparently unilocular units. The presence of a still spherical nucleus in these cells provides additional evidence in support of the conclusion that these cells are true multilocular brown fat cells and not unilocular white fat cells.

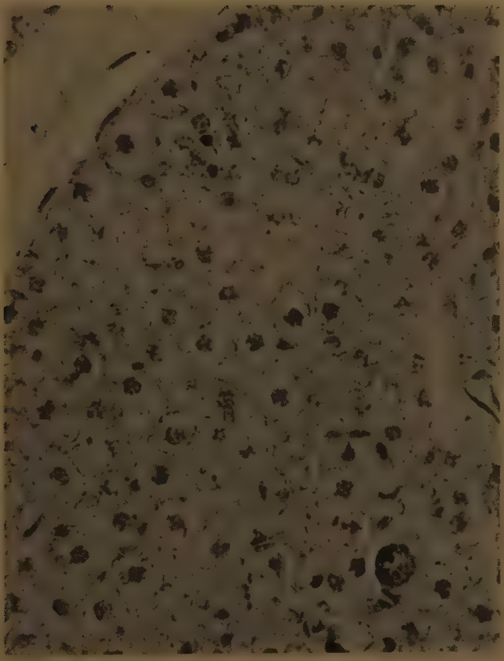

FIGURE 10. The shape of the multilocular brown adipose cells is demonstrated in this section stained with Masson's trichrome stain. Each cell has a densely chromatic nucleus and a cytoplasm packed with small lipid vacuoles. The capillary network surrounds individual cells of brown fat and outlines the polygonal shape of each cell clearly. × 350.

Such cells with very large lipid droplets were never found in equal abundance at any other period of the year in the interscapular lobes of *Myotis lucifugus lucifugus*. Another characteristic feature of the interscapular lobes collected during the summer months extending from May to the end of August is the "spotty" appearance of the "medulla." This is believed to be due to the variations in the number, size, and intensity of coloration of the lipid droplets from cell to cell within the medulla of a lobule. While certain medullary cells are laden with deeply colored droplets of various sizes (usually from 3 to 6 larger droplets and more numerous smaller droplets per cell), other medullary cells have a smaller number of slightly lighter colored droplets, and intermediates between these two most abundant types of cells can also be recognized.

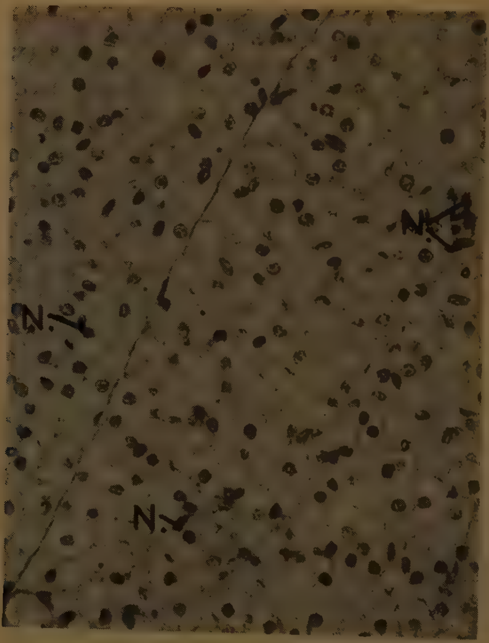

FIGURE 11. The shape of the cells of brown fat and the position and shape of the nuclei within a multilocular cell can be observed in this section treated by the Periodic Acid-Schiff test. A very faint (pinkish) tint indicative of glycogen is imparted to the nonvacuolar part of the cytoplasm. The darker-staining elongated nuclei represent the endothelial nuclei of the capillaries. × 240.
Symbol: *N* = nucleus of an endothelial cell.

The spotty appearance of these cells is very evident in sections of interscapular brown fat collected from May to the beginning of August (FIGURES 18 and 20). At the end of August, the spotty appearance has already started to fade, and a slightly more uniform picture is seen in the interscapular lobes (FIGURES 19 and 21).

Qualitative variations in the chemical composition of the lipid droplets of the brown fat cells. For the histochemical definition of lipids, certain specific colorants are used. Of these, Sudan black is by far the

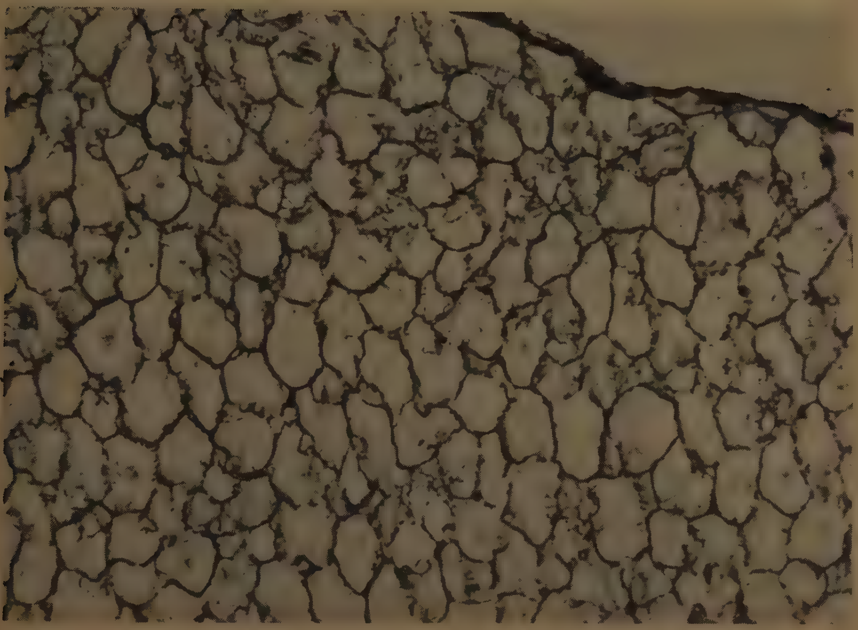

FIGURE 12. Section treated by Gömöri's silver oxide method for reticulum. The interscapular lobe is richly supplied with reticular fibers that surround each cell of brown fat. ×425.

most powerful. As pointed out by Lison (1953), the Sudans are not dyes in the strict sense of the word, and therefore they do not stain. They are colored compounds that color lipids by dissolving in them. Since these colored compounds are insoluble in water, but are soluble in fat solvents and in the fats themselves, they can be dissolved in a mixture of organic solvents and water. With an adequate proportion of organic solvents and water, the coefficient of partition of the Sudan black colorant between the dissolving mixture and the lipids is enormously in favor of the latter. In animal tissues the lipids are the only known substances whose coefficient of partition is highly in favor of fat-soluble colorants such as Sudan black. Consequently, the Sudan colorants are highly specific for lipids, and it is not believed that they color other substances.

This does not mean that all the objects colored with Sudan black are entirely of a lipid nature, or that all the lipids are colored with Sudan black. Solid lipids are not colored by the Sudan black colorants. Treating the sections with hot water (50 to 60° C.) dissolves some of the lipid inclusions and crystals (Lison, 1953), but a complete account of the solid lipids included in a tissue must involve a study of the tissue with the polarizing microscope. Therefore, the results obtained with Sudan black describe the localization of all the lipids encountered in a tissue, except

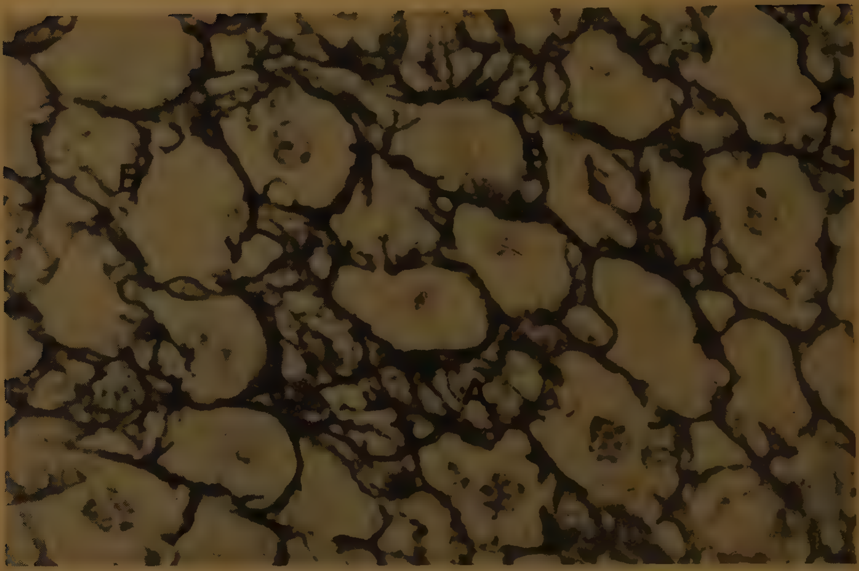

FIGURE 13. Higher magnification of the section shown in FIGURE 12. The basketlike arrangement of the reticular fibers around a cell of brown fat is shown at *A*. Blood capillaries are distributed through the meshes of the reticular network *B*. × 1100.

for the lipid crystals and solid inclusions that must be examined with the polarizing microscope.

In frozen sections of the interscapular brown fat treated with Sudan black B, the multiple fat droplets of this tissue are colored an intense bluish black, while the remainder of the cytoplasm has an extremely faint bluish gray tint that persists after a four-hour extraction of the sections with acetone, chloroform, ether, 95 per cent ethanol, or an ethanol-ether (3:1) mixture. It seems, therefore, that the cytoplasmic droplets of brown adipose cells are especially rich in lipids in the liquid state, while the remainder of the cell contains a much smaller amount of such lipids.

When frozen sections of either fresh brown fat tissues or tissues fixed in neutral formalin or formal-calcium are examined with the polarizing microscope, no definite birefringence can be observed in the sections. According to Lison (1953), no conclusion can be drawn from such negative results, and any lipid, whether in the liquid or in the solid state, can be present in the brown fat tissue. "The glycerides and the fatty acids, in their melted state, are never, as a matter of fact, birefringent; the cholesterides and the lipins may be, but are not always so, according to whether they are found above the point of clarity or whether some physical condition inhibits the formation of liquid crystals."* (Lison, 1953, p.366).

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*Editor's translation.

Thus, a comparison between the results obtained with the Sudan black method and those obtained with the birefringence tests indicates that lipids in the liquid state are present in brown adipose tissue of *Myotis lucifugus lucifugus*, but it is impossible to conclude whether or not solid inclusions or crystals of lipids are present in this tissue.

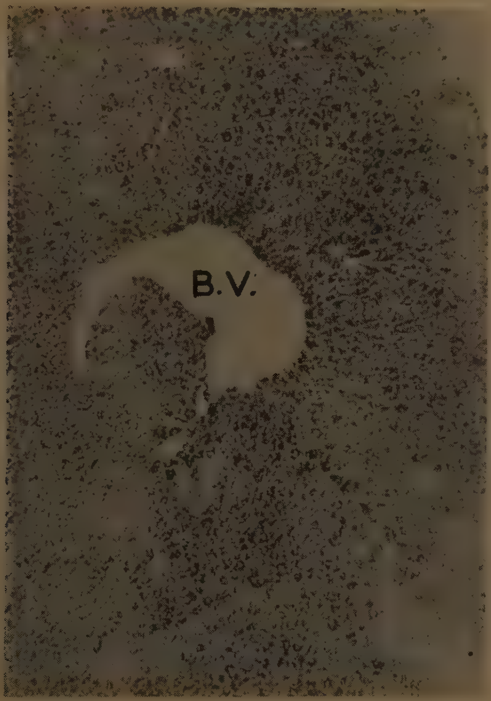

FIGURE 14. A rather uniform picture is seen throughout the whole extent of the lobules in this frozen section from an interscapular lobe of a specimen collected in December. Each cell is heavily laden with densely colored lipid droplets of uniform size. Treated with Sudan black B. × 100.

Symbols: *B.V.* = blood vessels; *S* = connective-tissue septa.

Further information can be gained on the chemical composition of the liquid lipids of brown fat tissue by staining the frozen sections with Nile blue sulfate. This dye consists of a mixture of a blue oxazine (Nile blue) and an impurity (Nile red) that probably is identical with the oxazone, a derivative obtained upon demethylation of the oxazine (Lison, 1935 and 1953). With the Nile blue sulfate method, the "neutral lipids" in the liquid state are colored pink by the oxazone, while the "acidic lipids" in the liquid state are colored blue by the oxazine form of the dye.

With the Nile blue sulfate solutions, the cytoplasmic droplets of brown fat tissues collected during the summer months took on the pink color of the oxazone form of the dye, while the remainder of the cell remained colorless (FIGURES 22 and 23). This reaction indicates that during the summer months the cytoplasmic droplets of the brown fat cells contain mostly neutral lipids, although traces of acidic lipids also may be present (Cain, 1947a). In animal tissues, the neutral lipids consist essentially of the esters of glycerol and the esters of cholesterol (Lison, 1953). However, tripalmitin, tristearin, and cholesterol are not detected by the Nile blue sulfate reaction, since these compounds are solid at ordinary temperatures (Cain, 1947a; Lison, 1953).

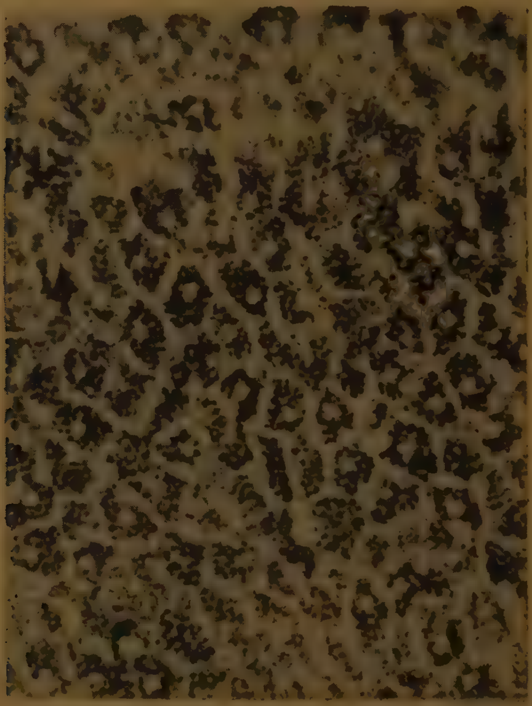

FIGURE 15. In this frozen section taken from a specimen collected in December, each cell of brown fat is heavily laden with lipid droplets of fairly uniform size. The blood capillaries are abundant, and they appear as irregular white spaces between the cells of brown fat. Treated with Sudan black B. ×350.

The cytoplasmic droplets of interscapular brown fat tissues collected during the winter months assume both the pink color of the oxazone and the blue color of the oxazine form of the dye when sections are treated with a dilute Nile blue sulfate solution. In the interscapular lobes collected in December (FIGURE 24), there is an irregular distribution of three "types" of multilocular cells: cells containing blue-colored droplets, cells containing pink-colored droplets, and a greater number of

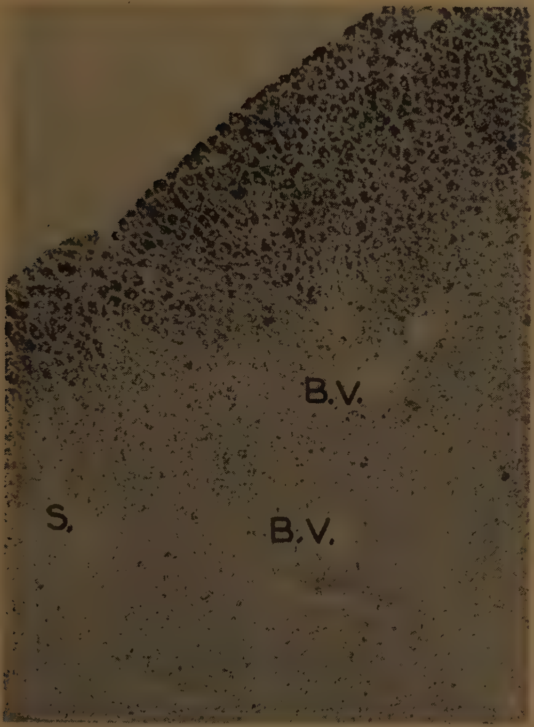

FIGURE 16. Frozen section from an interscapular lobe of a specimen collected at the end of the period of hibernation. Within a lobe a "cortical" region of cells of brown fat with densely packed and intensely colored lipid droplets can be distinguished from a "medullary" region that embodies cells of brown fat that contain fewer lipid droplets of lighter coloration. × 100.

Symbols: *B.V.* = blood vessels; *S* = connective-tissue septa.

cells containing a mixture of droplets of each color. In the lobes collected in March, a few lobules are filled almost entirely with cells containing blue-colored droplets, while most lobules are almost entirely filled with cells containing purple-colored droplets. Thus, it seems that there is some chemical transformation in the lipids of the cytoplasmic droplets during the winter months. The results obtained with the Nile blue sulfate method apparently indicate that the neutral lipids of the cytoplasmic droplets are transformed into acidic lipids during hibernation. These acidic lipids may consist of traces of oleic acid or other fatty acids in the liquid state, and lipins as well as chromolipoids may also be present in the cytoplasmic droplets during the hibernating period. With respect to the lipins, it seems that traces, at most, may be present. Although Cain (1948b) maintains that Nile blue cannot be used to distinguish among the various members of the lipins, the lecithins and the galactolipins seem

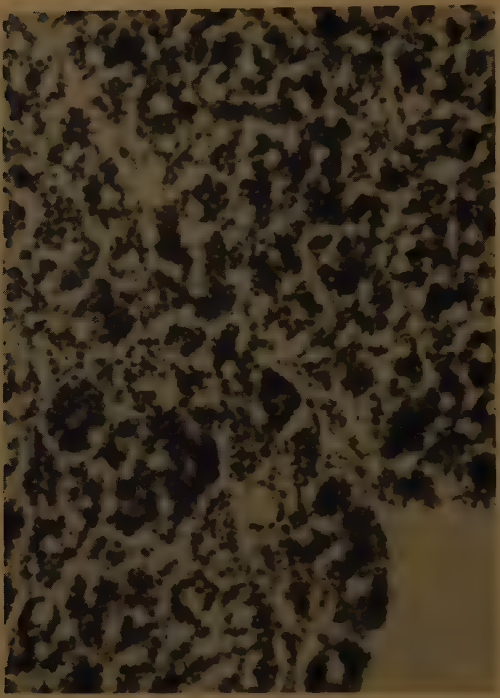

FIGURE 17. Frozen section through the "cortical" region of an interscapular lobe of *Myotis lucifugus lucifugus* collected at the end of the period of hibernation. Each cell of brown fat is densely packed with intensely colored lipid droplets of uniform size. Blood capillaries surround individual cells of brown fat and outline their shape. The site of the nucleus of each multilocular adipose cell appears as a round white spot close to the center of the cell. Treated with Sudan black B. × 350.

to belong to the group of "neutral" lipids (Lison, 1953). These lipin compounds may, therefore, be present in brown fat throughout the year, although their concentration seems to be higher in brown fat tissues collected during the summer months.

Throughout the year, the lipid droplets of the brown fat cells also were stained with some of the histochemical color tests for steroids. In Romieu's test (Lison, 1953), the fat droplets were first colored purple. Within 30 to 45 minutes, the purple color of the droplets had changed to green, while the other parts of the cell remained colorless. When the Schultz tests (Schultz, 1924), with oxidation, were applied to frozen sections of interscapular brown fat, the color changes just mentioned were observed. These color changes were also identical with those seen in control sections of the adrenal glands of mice that had been treated similarly. The positive reactions obtained in all these tests suggest that sterols, either free or esterified, are present in the cytoplasmic droplets of the brown fat cells of *Myotis lucifugus lucifugus* throughout the year. However, the negative results obtained with the digitonin histochemical tests imply that free sterols are absent from these cells. Indeed, the free hydroxyl group of the 3β-hydroxysterols is essential for a positive reaction in the digitonin test, and the negative results obtained with this test in the present investigation imply, therefore, that only esters of sterols are present in the brown fat tissues under study.

FIGURE 18. Frozen section from an interscapular lobe of *Myotis lucifugus lucifugus* collected at the end of May. A very narrow "cortical" region and a large "medullary" region can be recognized in this part of a section of a lobe. The spotty appearance of the "medulla" is a characteristic feature of the interscapular lobes collected during the summer months. Treated with Sudan black B. ×100. Symbols: *B.V.* = blood vessels; *S.* = connective-tissue septa.

Since cholesterol and its esters are by far the most abundant forms of steroids encountered in animal tissues (Cain, 1950; Lison, 1953), the results of these histochemical investigations on the steroid components of the interscapular brown fat of *Myotis lucifugus lucifugus* seem to indicate that esters of cholesterol are present in the cytoplasmic droplets of the brown fat cells studied. Moreover, the negative results obtained with the Schultz test without oxidation further imply that oxidation products of cholesterol are absent (Cain, 1950) from the tissues investigated in the present work.

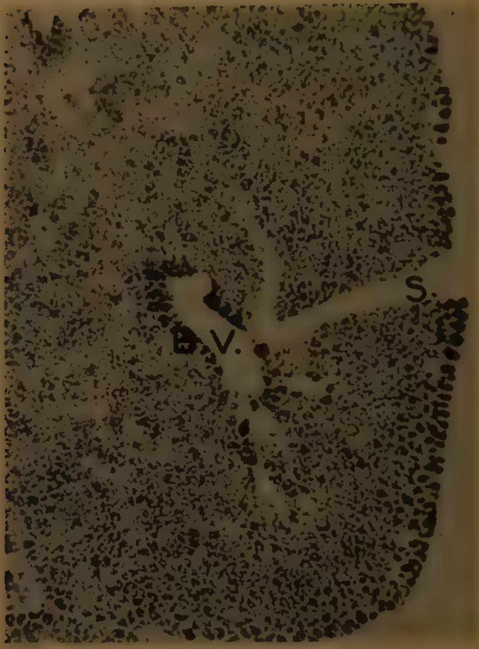

FIGURE 19. Frozen section from an interscapular lobe of a specimen collected at the end of August. A very narrow "cortical" region and a large "medullary" region still can be recognized. Moreover, very large lipid droplets are visible in the cytoplasm of some of the cells of brown fat. These droplets are in evidence anywhere within the lobules, but they seem to appear in greatest abundance in the cells in close proximity to the walls of the larger blood vessels and at the periphery of the lobules. Treated with Sudan black B. × 100.

Symbols: *B.V.* = blood vessels; *S.* = connective-tissue septa.

To summarize the various results obtained with the histochemical reactions of the cytoplasmic droplets of the interscapular brown fat cells of *Myotis lucifugus lucifugus*, it seems that:

During the summer months, these droplets consist of neutral lipids in the liquid state and, possibly, traces of acidic lipids and lipins such as galactolipins and lecithins. Solid lipids such as palmitic acid, stearic acid, tristearin, tripalmitin, and cholesterol could not be detected in the various histochemical tests used in the present work.

There appears to be an increase in acidic lipids in the cytoplasmic droplets during the period of hibernation. These acidic lipids may be either traces of liquid fatty acids or chromolipoids or certain lipins, or a mixture of any of these lipid components.

Throughout the year the cytoplasmic droplets are especially rich in cholesterol esters. This richness is implied by the fact that the histochemical color tests for steroids are very insensitive and will be positive

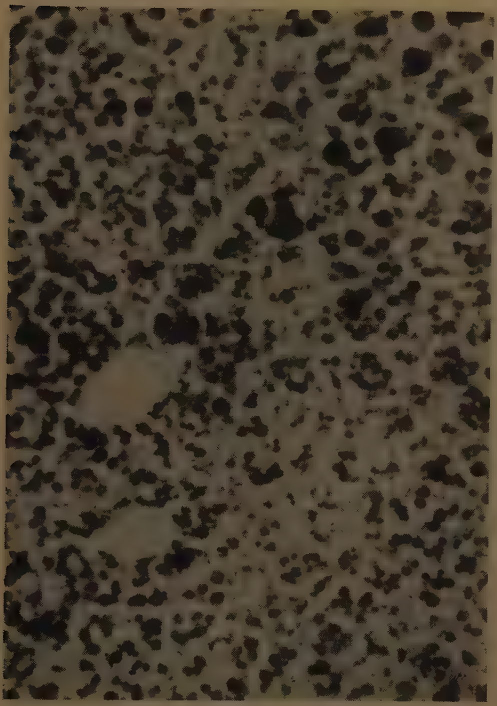

FIGURE 20. Very large lipid droplets are visible in some of the cells of brown fat in this frozen section from an interscapular lobe of *Myotis lucifugus lucifugus* collected at the end of May. These very large lipid droplets can be found in cells anywhere within the lobules, but the greatest abundance of them is seen at the periphery (upper right). Treated with Sudan black B. ×350.

only with concentrations of at least 40 to 50 per cent of cholesterol in the tissues (Reiner, 1953).

Structure and chemical composition of the cytoplasmic granules of the brown fat cell. Baker's acid hematein test is a modification of the Smith-Dietrich reaction and it is more complex, more sensitive, and more specific than the latter (Lison, 1953; Casselman, 1952).

Since the substances colored by the acid hematein test may be lipins as well as certain proteins, a control test involving pyridine extraction must be performed. Extraction of the sections with hot (60°C.) pyridine dissolves the lipids, including the lipins. Consequently, a substance that reacts positively with the acid hematein test before pyridine extraction and is negative after such an extraction can be considered as a lipin; a substance that is still colored by the acid hematein after pyridine extraction is presumed to be a protein.

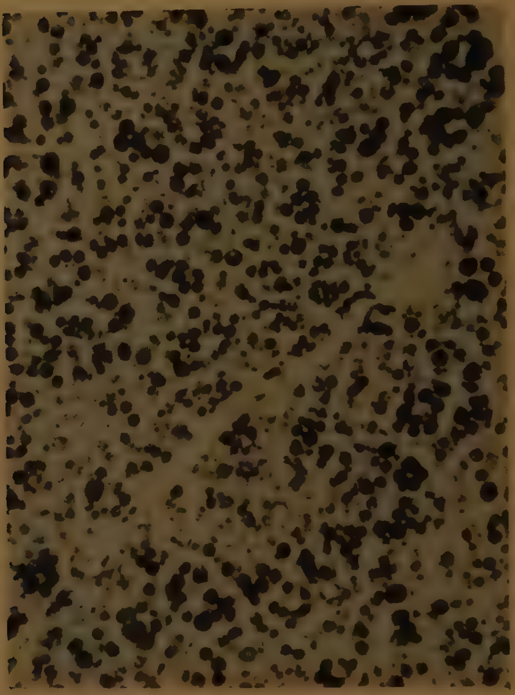

FIGURE 21. The variations in size, number, and coloration of the lipid droplets from cell to cell are visible in this section of an interscapular lobe taken from a specimen collected at the end of August. Treated with Sudan black B. ×350.

In frozen sections of interscapular brown fat treated by Baker's acid hematein test without pyridine extraction, the cytoplasmic lipid droplets of the brown adipose cells are colorless, while one or two dark blue dots can be seen in the very light brown nucleus. The remainder of the cytoplasm has a very light blue background and is filled with a few discrete, short rods and many tiny spherical granules that are interspersed between the lipid droplets. These granules and rods are seen to lie in close contact with the periphery of each lipid droplet and with the nuclear membrane (FIGURES 25 to 28).

In frozen sections of interscapular brown fat tissues that have been extracted with hot pyridine and treated subsequently with acid hematein, the nucleoplasm of the brown fat cell is colorless, and its nuclear chromatin is dark blue. The cytoplasm has a colorless background and is filled with very light grayish-brown granules and colorless vacuoles (FIGURE 29).

From the results obtained with the acid hematein tests with or without pyridine extraction, only two groups of cellular structures are thus

FIGURE 22. Frozen section from an interscapular lobe of *Myotis lucifugus lucifugus* collected in May. In this section, treated with a Nile blue sulfate solution, the cytoplasmic lipid droplets of the cells of brown fat took on the pink color of the oxazone form of the dye, while the other tissue structures remained colorless. ×450.

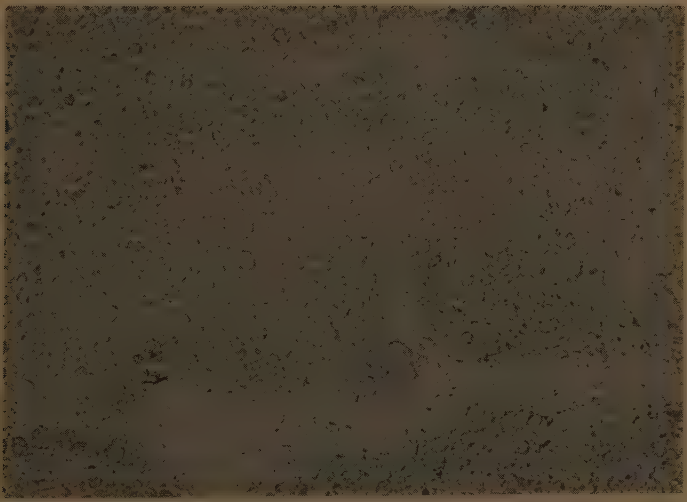

FIGURE 23. This frozen section of an interscapular lobe of a specimen collected in August was treated with a Nile blue sulfate solution. Very large lipid droplets are visible in the cells of brown fat that are in proximity to the walls of the larger blood vessels. The coloration of all of the structures in this section was similar to those observed in the lobes collected in May (FIGURE 22). ×450.

seen to give a positive reaction. While the cytoplasmic granules are stained only in the sections that have not been extracted with pyridine, the chromatin material of the nucleus is the only structure that gives a definitely positive, dark blue reaction after the extraction with pyridine. This coloration of the nucleus after pyridine extraction is of common occurrence in all tissues, and the mechanism involved in this phenomenon has not yet been explained (Lison, 1953). However, the presence of granules in the cytoplasm of the brown fat cell is revealed for the first time in this histochemical test, and the positive reaction of these granules with Baker's tests strongly suggests that they contain lipins.

FIGURE 24. The cytoplasmic lipid droplets of the cells of brown fat took on both the pink color of the oxazone form and the blue color of the oxazine form of the dilute Nile blue sulfate solution in this frozen section taken from an interscapular lobe of a specimen collected in December. There is an irregular distribution of three "types" of multilocular cells: (1) those that contain blue-colored droplets; (2) those that contain pink-colored droplets; and (3) those that contain a mixture of both. ×300.

The shape, number, and intensity of coloration of the cytoplasmic granules in the brown fat cell do not seem to vary throughout the year. It is therefore believed that the cytoplasmic granules of the brown fat cells of *Myotis lucifugus lucifugus* are especially rich in lipins throughout the year. The morphological appearance of these cytoplasmic granules further suggests that they might be mitochondria.

In an attempt to correlate the acid hematein-positive granules with mitochondria, a few additional staining reactions were studied. In sections embedded in Tissuemat and stained with Heidenhain's iron alum hematoxylin (FIGURE 30), or with Mallory's phosphotungstic acid iron hematoxylin (FIGURE 31), granular spherical inclusions of uniform size can be seen in the cytoplasm of the brown fat cells of *Myotis lucifugus lucifugus*. The distribution, size, and shape of these granular spherical inclusions seem to be identical to those of the granules observed in frozen sections of brown adipose cells treated with acid hematein (FIGURE 28). In addition, the cytoplasmic dark blue granules of the brown fat cells stained with acid hematein seem to correspond perfectly to the bright red mitochondria revealed by Cain's controlled method for staining mitochondria (FIGURE 32). From all these observations it seems that the cytoplasmic granules seen in the brown fat cells treated with Baker's acid hematein are mitochondria. It is believed, therefore, that the brown fat cells of *Myotis lucifugus lucifugus* are especially rich in lipins that are concentrated in tiny spherical cytoplasmic granules that seem to be mitochondria.

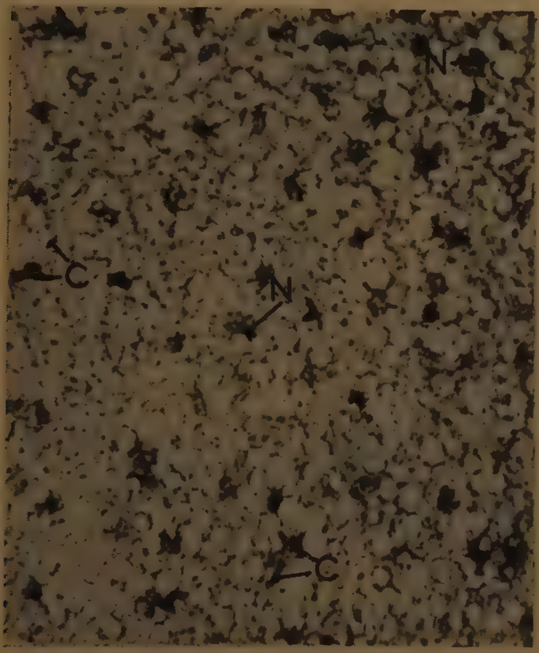

FIGURE 25. Frozen section of an interscapular lobe taken from *Myotis lucifugus lucifugus* collected in August and treated by Baker's acid-hematein test without pyridine extraction. Numerous granules and tiny rods are visible in close contact with the periphery of each droplet of colorless lipid. ×450.

Symbols: *C* = capillaries filled with blood cells; *N* = nucleus of a cell of brown fat.

Quantitative variations in the water content and the total solids of interscapular brown fat homogenates. The results obtained from the determinations of the amount of water and total solids in the brown adipose tissue of *Myotis lucifugus lucifugus* are listed in TABLES 2 and 3, and the averages for each group of samples are summarized in TABLES 4 and 5. In all of these tables, the analytical methods used for the determinations of water and total solids are specified: Method No. 1 refers to the method involving the evaporation of a tissue sample in an oven at 60° C. during twenty-four hours, while Method No. 2 refers to the evaporation of

TABLE 2

DETERMINATIONS OF WATER AND SOLIDS (OR DRY RESIDUE) IN THE INTERSCAPULAR BROWN FAT: ABSOLUTE VALUES

Number of group	*Total number of analytical determinations*	*Analytical method used*	*Volume of sample (in ml.)*	*Weight of solids in sample (in mg.)*	*Total weight of solids in brown fat (in mg.)*	*Total weight of water in brown fat (in mg.)*
1	4	1	2	8.1	101.2	203.3
		1	2	8.1	101.2	203.3
		2	2	9.0	112.5	192.0
		2	2	9.0	112.5	192.0
2	6	1	4	7.0	87.5	255.4
		1	4	6.9	85.2	257.7
		1	4	9.5	118.7	224.2
		1	4	9.3	116.2	226.7
		2	1	2.0	100.0	242.9
		2	1	2.0	100.0	242.9
3	5	1	4	9.8	122.5	4.6
		1	4	9.2	115.0	12.1
		2	1	2.3	115.0	12.1
		2	1	2.4	120.0	7.1
		2	1	2.0	100.0	27.1
4	4	1	4	9.9	123.7	9.9
		1	4	10.5	131.2	10.5
		2	1	2.4	120.0	2.4
		2	1	2.4	120.0	2.4
5	4	1	4	32.9	205.6	286.5
		1	4	32.8	205.0	287.1
		2	1	9.4	235.0	257.1
		2	1	8.7	217.5	274.6
6	4	1	4	14.7	91.9	68.5
		1	4	14.8	92.5	67.9
		2	1	3.9	97.5	62.9
		2	1	3.9	97.5	62.9
7	4	1	4	25.7	279.0	202.6
		1	4	28.6	311.0	170.6
		2	1	6.7	292.0	189.6
		2	1	6.5	283.4	198.2
8	4	1	4	19.6	245.0	187.0
		1	4	19.4	242.5	189.5
		2	1	5.9	295.0	137.0
		2	1	5.0	250.0	182.0
9	4	1	4	8.7	108.7	0.4
		1	4	8.7	108.7	0.4
		2	1	2.1	105.0	4.1
		2	1	1.9	95.0	14.1

an aliquot of the tissue homogenate at a constant temperature in the Abderhalden vacuum-drying apparatus.

The calculations of the percentages of water in the brown fat at different times of the year show clearly that the amount of water in brown fat varies throughout the year. The water content decreases markedly

TABLE 3

DETERMINATIONS OF WATER AND SOLIDS IN THE INTERSCAPULAR BROWN FAT: PERCENTAGES OF FRESH WEIGHTS

Number of group	*Total number of analytical determinations*	*Analytical method used*	*Per cent solids (mg. of solids per 100 mg. of brown fat)*	*Per cent water (mg. of water per 100 mg. of brown fat)*
1	4	1	33.2	66.8
		1	33.2	66.8
		2	36.9	63.1
		2	36.9	63.1
2	6	1	25.5	74.5
		1	25.2	74.8
		1	34.7	65.3
		1	33.7	66.3
		2	29.4	70.6
		2	29.4	70.6
3	5	1	96.1	3.9
		1	90.2	9.8
		2	92.0	8.0
		2	96.0	4.0
		2	80.0	20.0
4	4	1	90.0	10.0
		1	95.4	4.6
		2	88.9	11.1
		2	88.9	11.1
5	4	1	41.8	58.2
		1	41.7	58.3
		2	47.7	52.3
		2	44.2	55.8
6	4	1	57.0	43.0
		1	57.4	42.6
		2	60.9	39.1
		2	60.9	39.1
7	4	1	57.9	42.1
		1	64.6	35.4
		2	60.6	39.4
		2	58.8	41.2
8	4	1	56.6	43.4
		1	56.1	43.9
		2	67.8	32.2
		2	57.5	42.5
9	4	1	99.6	0.4
		1	99.6	0.4
		2	95.4	4.6
		2	86.4	13.6

TABLE 4

DETERMINATIONS OF WATER AND SOLIDS IN THE INTERSCAPULAR BROWN FAT: AVERAGE ABSOLUTE VALUES

No. of group	*Analytical method used*	*Solids (mg.)*	*Water (mg.)*	*No. of group*	*Analytical method used*	*Solids (mg.)*	*Water (mg.)*
1	1	106.8	197.7	6	A	94.9	65.5
2	1	101.9	241.0	6	B	97.5	62.9
2	2	100.0	242.9	7	A	295.0	186.6
3	1	127.5	10.1	7	B	287.6	193.9
3	2	120.0	17.6	8	A	243.8	188.2
4	1	118.8	8.3	8	B	273.0	159.0
4	2	111.6	15.5	9	A	108.7	0.4
5	1	205.3	286.8	9	B	100.0	9.1
5	2	226.3	265.8				

from the end of May (Groups 1 and 2) to the beginning of August (Groups 3 and 4). This drop in water content is followed by a sharp increase as the animal approaches the period of hibernation (Groups 5 and 6, collected in the last days of August). Hibernation is, in turn, accompanied by a gradual decrease (Groups 7, 8, and 9) that reaches a very low level at the end of hibernation (Group 9). These observations on the gradual decrease in water content during the hibernating period of *Myotis lucifugus lucifugus* are in disagreement with the findings of Aeby (1875) on the brown fat of the marmot, and the data of Carlier and Evans (1904) on the "hibernating glands" of European hedgehogs. Although the present results differ diametrically from the only available data on the brown fat tissues of hibernators, they correspond qualitatively to the reported general loss of water in the bodies of hibernating mammals (Dubois, 1896; Benedict and Lee, 1938).

No data could be found in the literature concerning the changes in water content of brown adipose tissue of hibernators during their awakened period in the summer. However, Benedict and Lee (1938) noted that the loss of water from the whole body of the marmot was higher during the summer than during the winter. In comparing the water content of brown fat in *Myotis lucifugus lucifugus* at different times during the summer (TABLE 5), it can be seen that the loss of water from May to the beginning of August is very close to that occurring from the end of August to the end of the period of hibernation. Although these results do not seem to agree with those of Benedict and Lee (1938), it must be borne in mind that the end of August corresponds to a time that is about a month before the onset of hibernation in *Myotis lucifugus lucifugus*. It is possible that quantitative measurements of the water content of interscapular brown fat collected at the end of September (when it is very difficult to collect representative numbers of *Myotis lucifugus lucifugus*) would show that the interscapular tissue undergoes greater losses during the awakened period than during the winter months. Such results would thereby corroborate the findings of Benedict and Lee (1938) on the loss of water in the whole body at different periods of the year.

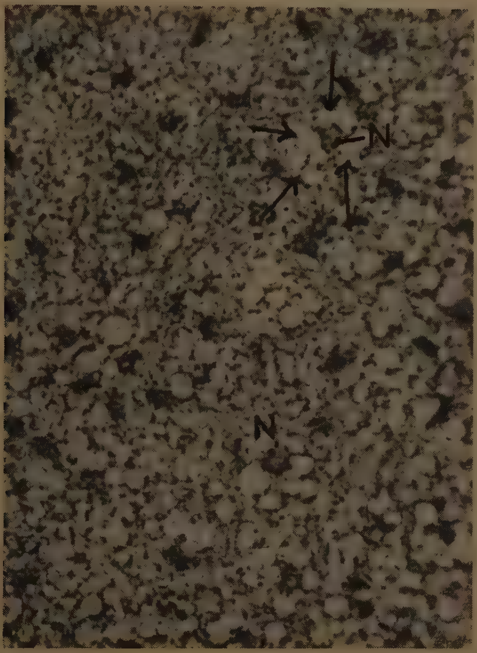

FIGURE 26. Frozen section of an interscapular lobe taken from a specimen collected in March and treated by Baker's acid-hematein test without pyridine extraction. The numerous tiny rods and granules are visible in close contact with the periphery of each droplet of colorless lipid and with the nuclear membrane.

Symbols: *N* = nucleus of a cell of brown fat; → indicates the nuclear membrane of the cell. ×450.

TABLE 5

DETERMINATIONS OF WATER AND SOLIDS IN THE INTERSCAPULAR BROWN FAT: AVERAGE VALUES EXPRESSED AS PERCENTAGE OF FRESH WEIGHTS

No. of group	*Analytical method used*	*Solids (per cent)*	*Water (per cent)*	*No. of group*	*Analytical method used*	*Solids (per cent)*	*Water (per cent)*
1	1	35.1	64.9	6	A	57.2	42.8
2	1	29.8	70.2	6	B	60.9	39.1
2	2	29.4	70.6	7	A	61.2	38.8
3	1	92.7	7.3	7	B	59.7	40.3
3	2	88.9	11.1	8	A	56.3	43.7
4	1	94.1	5.9	8	B	62.6	37.4
4	2	89.3	10.7	9	A	99.6	0.4
5	1	41.8	58.2	9	B	90.9	9.1

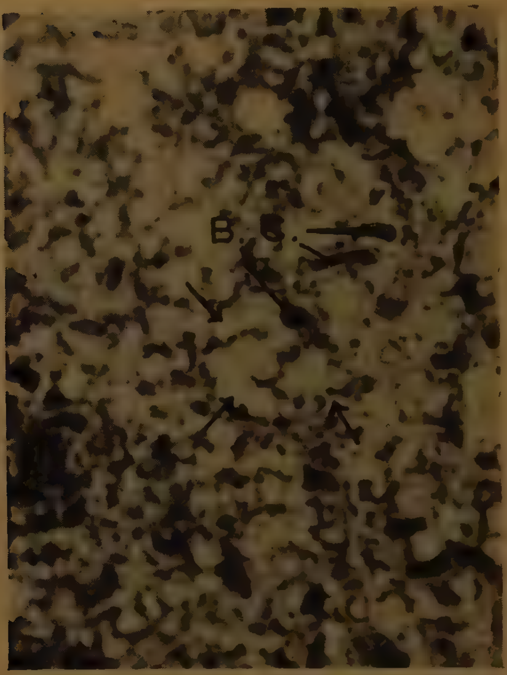

FIGURE 27. Higher magnification of a frozen section taken from an interscapular lobe of *Myotis lucifugus lucifugus* collected in August (FIGURE 25). This section was treated by Baker's acid-hematein test without pyridine extraction.

Symbols: *B.C.* = blood cells in the capillaries; → indicates the typical distribution of the cytoplasmic granules and rods. ×1230.

Quantitative variations in the total lipids of the interscapular brown fat homogenates. The data from the biochemical determinations of the total lipids in the brown adipose tissue extracts of *Myotis lucifugus lucifugus* are listed in TABLES 6, 7, and 8. The results obtained from the determinations of the absolute weights of the total lipids in the extracts are summarized in TABLE 6. From these results, the percentages of total lipids in the fresh tissues were calculated and summarized in TABLE 7.

In terms of percentages of the fresh weights of the tissues (TABLE 7), the amounts of total lipids in the extracts attain a minimum at the end of August (Groups 5 and 6) and a maximum at the beginning of August (Groups 3 and 4). However, more significant observations are made when the amount of water in the brown fat tissues is taken into consideration. In the latter case, the percentages of total lipids can be studied in terms of percentage of the dry weight or percentage of the total solid residue of the brown adipose tissue (TABLE 8).

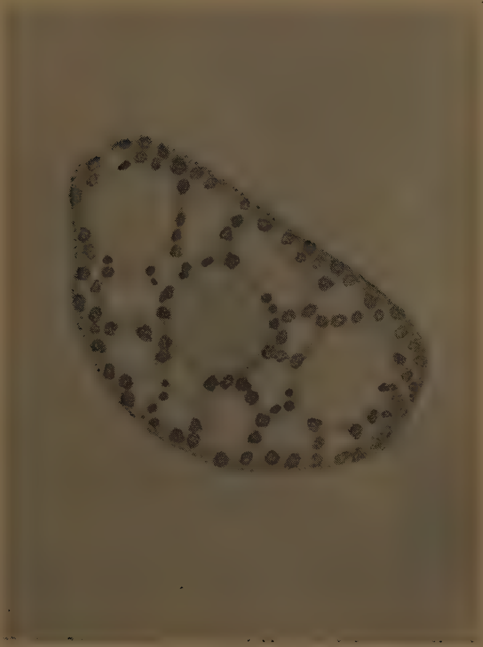

FIGURE 28. Drawing of a multilocular adipose cell from a section treated by Baker's acid-hematein test without pyridine extraction. The acid-hematein-positive, phospholipid-rich granules in the cytoplasm are visible in close contact with the nuclear membrane and with the periphery of the cytoplasmic lipid droplets. ×900.

TABLE 6

DETERMINATIONS OF THE TOTAL LIPIDS IN THE INTERSCAPULAR BROWN FAT

No. of group	*Weight of the total lipids in the ethanol-ether extract of brown fat (in mg.)*	*Weight of the total lipids in the acetone extract of brown fat (in mg.)*	*Weight of the total lipids in the whole extract of brown fat (in mg.)*
1	18.43	30.18	48.61
2	17.61	32.98	50.59
3	20.64	39.05	59.69
4	14.88	32.28	47.16
5	20.00	26.04	46.04
6	11.61	6.20	17.81
7	11.97	40.62	52.59
8	10.57	51.27	61.84
9	12.18	24.99	37.17

TABLE 7

DETERMINATIONS OF THE TOTAL LIPIDS IN THE INTERSCAPULAR BROWN FAT: PERCENTAGE OF THE WEIGHTS OF THE FRESH TISSUES*

No. of group	*Percentage of total lipids in the ethanol-ether extract of brown fat*	*Percentage of total lipids in the acetone extract of brown fat*	*Percentage of total lipids in the whole extract of brown fat*
1	6.05	9.93	15.98
2	5.13	9.62	14.75
3	15.00	28.38	43.38
4	11.71	25.40	37.11
5	4.06	5.29	9.35
6	7.24	3.86	11.10
7	2.48	8.43	10.91
8	2.45	11.87	14.32
9	11.16	22.90	34.06

*In mg. of total lipids per 100 mg. of tissue.

The total lipids constitute approximately one half (from 45.51 to 50.59 per cent) of the dry residue of brown fat collected at the beginning of the summer period. The concentration of total lipids in the dry residue subsequently decreases during the summer. At the end of August this concentration represents approximately one fifth (18.27 to 22.42 per cent) of the total dry residue of the brown fat tissues. This small amount seems to remain unchanged during the first half of the hibernating period. As the animal approaches the end of hibernation, the total lipids seem to increase in concentration in the dry residue of brown adipose tissues.

TABLE 8

DETERMINATIONS OF THE TOTAL LIPIDS IN THE INTERSCAPULAR BROWN FAT: PERCENTAGE OF THE TOTAL SOLIDS DETERMINED BY METHODS A AND B

No. of group	*Percentage of total lipids in the total solids of brown fat**	
	as determined by Method 1	*as determined by Method 2*
1	45.51	—
2	49.65	50.59
3	46.81	49.74
4	39.70	42.26
5	22.42	20.34
6	18.77	18.27
7	17.83	18.28
8	25.36	22.65
9	34.19	37.17

*In mg. of total lipids per 100 mg. of solids.

FIGURE 29. Frozen section of an interscapular lobe treated by Baker's acid-hematein test after extraction with hot pyridine. The nuclear chromatin is the only structure within a cell of brown fat that is intensely colored. The remainder of the cytoplasm and the nucleus of the cells of brown fat have a light gray color. ×450.

It therefore seems that the concentration of total lipids in the dry residue of the interscapular brown fat reaches its peak at the beginning of the awakened period when the animal is in full activity. This total lipid concentration is gradually lowered during the summer months to a value that is maintained during the first half of the hibernating period. Subsequently, the proportion of total lipids in the dry residue of brown fat apparently increases in preparation for the awakened period.

Quantitative variations in the total cholesterol of the interscapular brown fat homogenates. The results of the biochemical determinations of the total cholesterol in the brown fat extracts of *Myotis lucifugus lucifugus* are listed in TABLE 9. These data show that the amount of total cholesterol is low at all times of the year.

In terms of the percentages of the total cholesterol in the fresh tissues, the variations in the total cholesterol levels are seen to parallel those of the total lipids. However, when the amount of water is taken into consideration, and the concentration of total cholesterol is calculated in terms of percentages of the dry weight of the tissues, the levels of total cholesterol do not always parallel those of the total lipids.

Thus, while the total lipid concentration in the dry residue of interscapular brown fat decreases constantly during the summer months, the levels of total cholesterol in the dry residue seem to fluctuate. It is interesting to note, however, that the cholesterol levels during the summer months increase or decrease as the water content drops or rises.

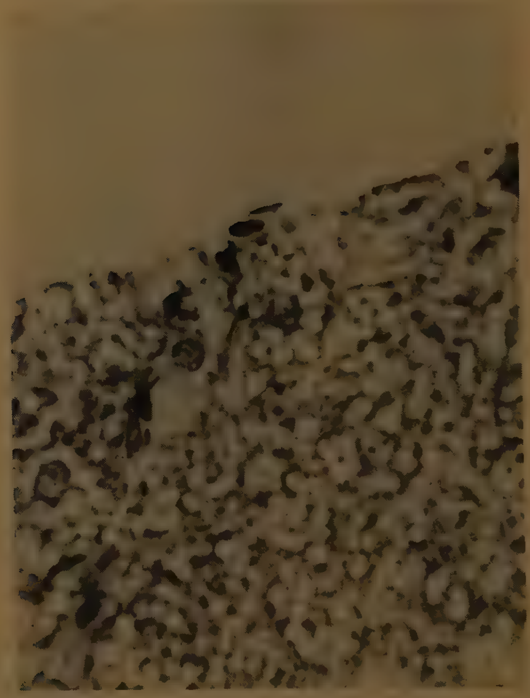

FIGURE 30. Section of an interscapular lobe stained with Heidenhain's iron alum hematoxylin to show the granular inclusions of uniform size in the cytoplasm of the cells of brown fat of *Myotis lucifugus lucifugus*. ×1250.

During the first half of the period of hibernation, there seems to be a depletion in the concentration of total cholesterol in the interscapular brown fat. This decrease is apparently followed by a rise in the level of total cholesterol during the second half of the period of hibernation.

A considerable amount of research is necessary before reaching any conclusion on the variations in the total lipid and total cholesterol levels of the interscapular brown fat of *Myotis lucifugus lucifugus*. However, the data recorded in the present investigation seem to indicate that, during hibernation, there is a reduction in the weight of the interscapular lobes

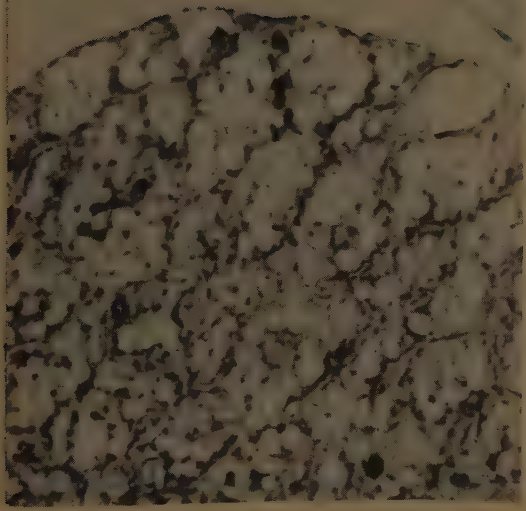

FIGURE 31. Section of the tissue shown in FIGURE 30, stained with Mallory's phosphotungstic acid iron hematoxylin to show the same granular inclusions in the cytoplasm of the cells of brown fat. ×1250.

TABLE 9

DETERMINATIONS OF THE TOTAL CHOLESTEROL IN THE INTERSCAPULAR BROWN FAT

No. of group	*Absolute weight of total cholesterol in the brown fat extracts (in mg.)*	*Percentage of total cholesterol in the fresh tissues**	*Percentage of total cholesterol in the dried tissues†*	
			Method 1	*Method 2*
1	1.58	0.52	1.48	—
2	2.22	0.65	2.18	2.22
3	4.94	3.59	3.87	4.11
4	4.66	3.67	3.93	4.18
5	2.29	0.78	2.41	2.35
6	2.50	0.85	2.63	2.56
7	1.31	0.34	0.44	0.46
8	1.41	0.42	0.58	0.52
9	1.45	1.33	1.34	1.45

*In mg. of total cholesterol per 100 mg. of fresh tissue.

†In mg. of total cholesterol per 100 mg. of dried tissue.

(TABLE 1), and that this is accompanied by a depletion of water in the brown adipose tissue (TABLE 5). The total cholesterol is also decreased in concentration, but this depletion occurs chiefly during the first half of the period of hibernation. As the animal approaches the end of hibernation, the total cholesterol levels are elevated slowly. This increase may correspond to a preparation for the awakening, which is accompanied by a higher rate of metabolism and an intensified sexual activity.

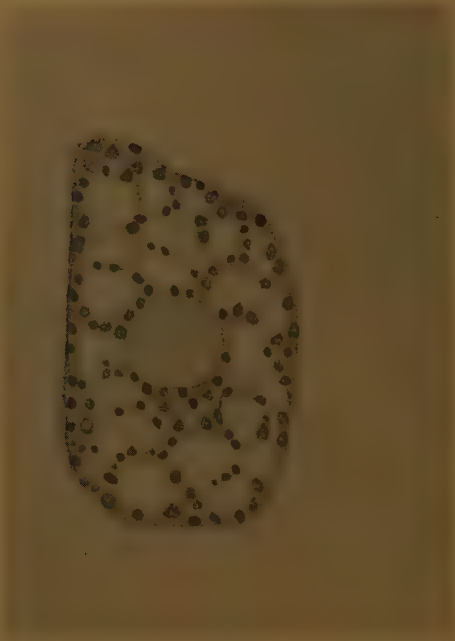

FIGURE 32. Drawing of a multilocular adipose cell from a section treated by Cain's (1948a) controlled method for staining mitochondria. The size, shape, and distribution of the cytoplasmic granules in this cell seem to correspond to those of the granules shown in FIGURE 28. ×900.

The data obtained from tissues collected during the summer months are even more difficult to interpret. These data may be related to the hibernating and awakened states, or they may simply reflect incidental changes in part, or in all, of the metabolism.

Quantitative variations in the total fatty acids of the interscapular brown fat homogenates. The data from the calculations of the concentrations of the total fatty acids in brown fat are listed in TABLE 10. These results show that the levels of total fatty acids in the brown adipose tissues of *Myotis lucifugus lucifugus* closely parallel the levels of the total lipids in these tissues. Consequently, the highest concentration of fatty acids is found in tissues collected at the beginning of the summer period. This high level decreases slowly during the summer to reach a minimum at the end of August. In turn, this minimum is maintained during the first half of the period of hibernation, and there is a subsequent rise in the amount of fatty acids during its second half.

Quantitative determinations of the total phosphorus content of the homogenates of interscapular brown fat. No detectable amounts of phosphorus could be found in the brown adipose tissues of *Myotis lucifugus lucifugus* with Tsuji's (1950) modification of Sumner's quantitative method for the determination of phosphorus.

TABLE 10

DETERMINATIONS OF THE TOTAL FATTY ACIDS IN THE INTERSCAPULAR BROWN FAT

No. of group	*Absolute weight of total fatty acids in the brown fat extracts (in mg.)*	*Percentage of total fatty acids in the fresh tissues**	*Percentage of total fatty acids in the dried tissues†*	
			Method 1	*Method 2*
1	16.85	15.46	44.99	–
2	15.39	14.10	47.47	48.37
3	15.70	39.79	42.94	45.63
4	10.22	33.44	35.77	38.08
5	17.71	8.57	20.01	17.99
6	9.11	10.25	16.14	15.71
7	10.66	10.57	17.39	17.82
8	9.16	13.90	24.78	22.13
9	10.73	32.73	32.85	35.72

*In mg. of total fatty acids per 100 mg. of fresh tissue.

†In mg. of total fatty acids per 100 mg. of dried tissue.

DISCUSSION

The histochemical and biochemical observations on the lipids of the interscapular brown fat of the female vespertilionid bat *Myotis lucifugus lucifugus* are discussed in the following section. Since the histochemical reactions of the lipids in the brown fat tissue of a hibernating mammal have been investigated for the first time in the present study, the results are discussed in the light of the observations reported by workers who have studied the brown fat of nonhibernating mammals. The biochemical data also are discussed, and they are compared to the scarce and often conflicting results reported in the literature dealing with the biochemical analyses of brown fat in hibernating animals.

Numerous workers have analyzed the changes in the total water content of hibernators. From these observations it seems that the absolute loss of water is appreciable during hibernation (Dubois, 1896; Rasmussen and Rasmussen, 1917; Benedict and Lee, 1938), but this decrease in water content is relatively lower than that encountered during the awakened period (Benedict and Lee, 1938). If the general loss of body weight is taken into account, the relative water content is even found to increase from November to April (Rulot, 1902; Mann, 1936).

The brown fat tissues of hibernating mammals also undergo seasonal variations in water content. Although no data could be found in the literature on biochemical analyses of the interscapular brown fat during the awakened period, a few analyses of the water content of this tissue during hibernation have been recorded. Aeby (1874) observed that the brown fat of marmots contained a slightly larger percentage of solids at the beginning than at the end of hibernation. This percentage dropped from 46.10 at the onset of hibernation to 45.50 at the end of the hibernating period. Carlier and Evans (1904) found an even greater difference between the beginning and the end of hibernation. Upon chemical analysis of the "hibernating glands" of hedgehogs *(Erinaceus europaeus)*, they observed that the percentage of moisture increased and that, consequently, the percentage of solids decreased, from October to April. Thus, there was 40.15 per cent water in the "glands" taken from animals at the beginning of October, while an increase to 60.44 per cent water was noted in "glands" taken from hedgehogs at the end of April. In the present experiments with *Myotis lucifugus lucifugus*, the percentage of water in the interscapular brown fat decreased from the end of August to the end of April (TABLE 3). These results are therefore in disagreement with those of Aeby and of Carlier and Evans. In order to account for these different results, two main factors should be considered, namely, the animals used in the experiments and the analytical methods employed.

Carlier and Evans worked with hedgehogs, and Aeby used two marmots, while the vespertilionid bat *Myotis lucifugus lucifugus* was used in the present study. Results obtained with different species and even different orders of mammals should be compared with extreme care.

The methods used for the determination of the water content of tissues can also be a source of error in analytical procedures. Aeby (1874) used only one marmot in each of his two analyses. Thus the very important influence of individual variations was overlooked, and this is well illustrated in a few cases listed in TABLE 2 of the present study, where variations can be noted between groups of animals collected at the same time of the year. In the experiments of Carlier and Evans (1904), "some twenty or thirty hedgehogs . . . were kept in a cool, quiet cellar on a diet of bread and milk, and allowed to remain undisturbed till required." These authors add the interesting observation that the animals were "but light sleepers in captivity." Without specifying the number of animals used for each analysis, Carlier and Evans state that, in their tables, "the mean values only are given, as greater accuracy is thereby probably obtained," and they further neglect to describe how the tissues were dried to constant weight.

The bats used in the present experiments were deep hibernators and, for the short period during which they were kept in captivity, they were allowed no food. Thus these experimental conditions differ markedly from those employed by Carlier and Evans (1904). In assuming that the latter workers noticed some variations in the percentage of water content of the "glands," but neglected those variations "to obtain greater accuracy" with mean values, it still remains to be proved that their analytical methods permitted such a use of mean values. Thus, in my experiments two methods were used for the determination of water content in the interscapular brown fat. The method involving desiccation in an oven at 55 to 60° C. often proved especially misleading. It was often noticed that the constant weights obtained after several weighings within a 12-hour desiccating period were only apparently constant; upon additional desiccation, greater amounts of water could often be removed from the tissues. The relatively large volume (4 ml.) of tissue homogenate used in each analysis and the large surface area of the containers proved to be additional sources of error because of the relatively large amounts of contamination.

In order to minimize those sources of errors, a careful drying technique was also used in each case. With the Abderhalden drier, the tissues were desiccated in a closed chamber under constant conditions of temperature, pressure, and humidity. This desiccating procedure was also more rapid than the one involving desiccation in a paraffin oven, and the values obtained with the Abderhalden technique could easily be duplicated. These advantages proved especially important when working with such small aliquots (1 ml.) as those used in the present investigation. Even with such a careful technique, such appreciable variations were observed between groups collected at the same period of the year (see TABLE 3) that mean values could not be used.

The relatively high values obtained by Carlier and Evans (1904) for the water content of the "hibernating glands" collected at the end of April may therefore be due to variations in the environmental conditions of temperature, pressure, and humidity. One must also consider the fact that the hedgehogs they used were still hibernating at the end of April, and it is possible that an analysis of the "glands" at a later date would have given results comparable to those that I obtained. This possibility is partly implied by Carlier and Evans themselves when they admit that "the gland is completely exhausted and reduces to a mere fibrous cord by the time the animal wakens into activity" (Carlier and Evans, 1904). It seems, therefore, that the differences between my results and those obtained by Carlier and Evans can be attributed to three main causes: first, these workers did not study the same mammalian order as the one used in the present work; second, their animals were in a different physiological state with respect to hibernation; and third, their experimental conditions differed from those encountered in the present investigation. Their analytical methods appear to be different from mine, but since the description of their technique is lacking, a critical comparison of their data as compared with mine is impossible.

The loss in weight during hibernation has long been explained as being due chiefly to the depletion of fat in the hibernating mammal. The first experimental proof of the importance of the reserves of fat in hibernation was given by Regnault and Reiset (1849), who observed that the respiratory quotient of hibernating mammals varied between 0.3 and 0.6. These low respiratory quotient (R.Q.) values were attributed to the partial oxidation of fats. That this may take place during hibernation was suggested by a tendency to acidosis brought about by the acids produced by the partial combustion of fats.

The high levels of glycogen observed in hibernating animals during their winter sleep were also thought to be the result of a partial oxidation of the body fats. Consequently, the R.Q. values below 0.7 encountered during hibernation were long used as a basic argument to prove that the mammalian organism could convert lipids into carbohydrates. More recently, however, the R.Q. values below 0.7 encountered during the hibernating period have been proved to be artifacts caused by measurements of respiratory quotients by the closed method (Dontcheff and Kayser, 1935b; Benedict and Lee, 1938), and the average R.Q. during hibernation was shown to be approximately 0.7 (Kayser, 1940b). This suggests that the lipids are the principal factor in the maintenance of the hibernating animal during its period of hibernation. The present investigation shows that brown fat is an especially rich source of various lipids that vary in nature and composition during the year. These observations, therefore, would imply that brown adipose tissues, as well as white fat, play a major role in the metabolism of the hibernating mammal.

To my knowledge, the quantitative changes in the lipids of brown fat during an entire year have never been investigated. However, Carlier and Evans (1904) have followed these changes in the "hibernating glands" of the hedgehog during the hibernating period. These workers observed a rapid fall in the weight of the "hibernating gland" during the first month of hibernation. Much fat was removed from the "gland" during this period. Subsequently, the "hibernating gland" was economized, "very little fat being removed from it until the end of March" (Carlier and Evans, 1904). In the present investigation, a great loss in the weight of the interscapular lobes was also noted (TABLE 1), and very little change in the amounts of total lipids and of total fatty acids could be observed from the end of August to December in the brown fat of *Myotis lucifugus lucifugus* (Groups 5-8 in TABLES 7 and 9). However, the data obtained by Williams (unpublished results) strongly suggest that the amount of total lipids and total fatty acids reaches a peak at the beginning of the hibernating period. It therefore seems that the percentage of total lipids and total fatty acids increases rapidly in the last month before the onset of hibernation, and that this increase is followed by a drop in the concentration of total lipids and total fatty acids during the first months of the hibernating period.

The quantitative changes in lipid content are accompanied by morphological alterations in the size of the brown fat cells. The decrease that takes place in these cells and in the whole organ from the beginning to the end of hibernation can be accounted for by the loss of fat during this period. This confirms the observations made on the brown fat of marmots (Rasmussen, 1923) and of hedgehogs (von Hansemann, 1902). Von Hansemann also noted that no such seasonal variations occurred in nonhibernators such as the rat, provided they remained well nourished.

No observations could be found in the literature concerning the histochemistry of the lipids in the brown fat of hibernators. However, Fawcett (1952) has studied the cytochemical reactions of brown adipose tissue in rats and mice. Although Fawcett's work does not cover observations on the brown fat of these nonhibernating mammals over the period of one year, a general comparison between his observations and mine can be made. His results with the Sudan black B method are similar to mine: in rats and mice, as well as in *Myotis lucifugus lucifugus*, the droplets of brown fat are colored an intense black, while the remainder of the cytoplasm reveals a faint blue-gray tint.

The strongly positive reaction of the cytoplasmic droplets indicates the presence of lipids (Lison, 1953), but the faint reaction of the remainder of the cytoplasm is difficult to explain. This light coloration persists after extraction of the sections with acetone, chloroform, ether, 95 per cent ethanol, an ethanol-ether mixture, or hot (37° C.) pyridine. Fawcett (1952) believes that the persistence of the coloration after an acetone extraction of the sections is due "to the phospholipid content of the mitochondria." In my opinion, the reaction of the cytoplasm after an acetone extraction of the sections cannot be used as an exclusive criterion for the presence of phospholipids, since these phospholipids are partly soluble in acetone, especially when they are associated with other lipids or cholesterol (Lison, 1953). The insoluble cytoplasmic components revealed after an acetone extraction of the sections and a Sudan black staining may very well be other lipids such as the chromolipids, which are quite insoluble in acetone. The persistence of the coloration of the cytoplasm after a pyridine extraction of the sections even suggests that this coloration is due to substances that are not of a purely lipid nature (Cain, 1947a). Therefore, no definite condlusion on the cytochemical composition of the "extravacuolar" part of the cytoplasm of the brown fat cell can be drawn from the various extraction tests followed by Sudan black staining. However, a correlation between these results and those obtained with the acid hematein tests merely suggests that the substances responsible for the slight sudanophilic reaction of the cytoplasm of the brown fat cell might be phospholipids, perhaps the "structural phospholipids of Sinclair" (1935).

With the Nile blue sulfate reaction, the colorations of the brown fat cells of rats and mice (Fawcett, 1952) can be compared only to the colorations of the brown adipose tissue cells of *Myotis lucifugus lucifugus* collected during the summer months. It seems that neutral lipids and traces of acidic lipids (Cain, 1947a) are present in the cytoplasmic droplets of the brown fat cell of the bat at this time of the year. However, when dilute solutions of Nile blue sulfate are applied to brown fat tissues of bats collected during their hibernating period, the reactions of the cells of *Myotis lucifugus lucifugus* are different from those observed in the brown fat cells of rats and mice (Fawcett, 1952): some lipid droplets of the brown fat cells of the hibernating bat take the blue color of the oxazine form of the dye, while others are colored pink by the oxazone form of Nile blue sulfate. It thus seems that such reactions represent changes in the chemical composition of the lipids of the cytoplasmic droplets during hibernation, and it is believed that there is an increase in acidic lipids in these droplets during hibernation. These acidic lipids may be free fatty acids in the liquid state (principally oleic acid and other unsaturated fatty acids), certain phospholipids, chromolipids, or a mixture of any of these acidic lipids (Cain, 1947a; Lison, 1953).

Since the blue color obtained by the Nile blue reaction is not specific for lipids (Lison, 1953), it may be argued that the blue color observed in some cytoplasmic droplets of the brown fat cells during hibernation is due to other biochemical components of the cells, such as proteins. Cain (1947a) suggests that "the only safe method for determining whether a body is purely lipoid is to color pyridine-extracted sections with Sudan black and see if it has been completely removed." In treating pyridine-extracted sections with Sudan black B, the cytoplasmic droplets were completely colorless, while the light grayish tint of the remainder of the cytoplasm persisted. The lack of coloration of the lipid droplets in this test therefore adduces additional evidence in favor of the presence of pure lipids of an acidic nature in the cytoplasmic droplets of brown fat cells during the hibernating period.

The birefringence tests gave negative results when applied to frozen sections of brown fat tissues of *Myotis lucifugus lucifugus*. The failure to detect some anisotropic material in the interscapular brown fat of the bat leads to no possible conclusion, and it should be emphasized that "negative results mean nothing" (Cain, 1950). Therefore, the negative results obtained in my investigation neither confirm nor contradict the observations noted in my other histochemical tests. Fawcett (1952) observed that the birefringence occasionally seen in the brown fat of rats and mice was "not in the form of a Maltese cross and [that] it disappears quickly upon warming," and he adds that the birefringence appears to be due to "crystals of fatty acid or neutral fat." This interpretation is in agreement with Lison's (1953) conclusion that if the birefringence of the crystals disappears upon warming and spherocrystals are not produced, "one can presume but not ascertain the absence of cholesterides or lipins," and that in this case crystals of fatty acids or neutral fats may be present. Thus, although Fawcett (1952) obtained partially positive results, they do not lead to any definite conclusion and therefore do not contradict my own conclusions. However, the observations of Baker, Ingle and Li (1950) are in disagreement with those of Fawcett (1952) and with my own.

In the brown fat of rats, Baker, Ingle, and Li (1950) observed the presence of abundant birefringent material that was concentrated mostly in the central cells of the lobules. A few spherocrystals were seen upon cooling the sections, and these authors say that this finding "might be suggestive of the presence of cholesterol or its esters." This interpretation of Baker, Ingle, and Li must be corrected in part. Although it is true that cholesterol esters form spherocrystals, free cholesterol crystallizes in the form of birefringent rhombic plates (Lison, 1953). Since the presence of spherocrystals excludes only the possible occurrence of free cholesterol in the tissues investigated by Baker, Ingle, and Li, it seems that these workers at least observed the presence of cholesterol esters in the brown adipose cells of rats. However, their Schultz tests for cholesterol esters gave negative results. Therefore it is believed that the spherocrystals observed by Baker, Ingle, and Li may actually represent phospholipids, which also form liquid crystals (Lison, 1953).

Since the studies of frozen sections by means of the polarizing microscope failed to reveal the presence of anisotropic material in the brown fat of *Myotis lucifugus lucifugus*, several other histochemical tests were made in an attempt to detect steroids in the brown adipose tissue of this hibernating vespertilionid bat. The negative results obtained with the Schultz tests (without oxidation) suggest that oxidation products of steroids are absent (Cain, 1950). However, esters of sterols (principally cholesterol esters) are probably present in the brown fat tissues of *Myotis lucifugus lucifugus*, since the Schultz tests (with oxidation) and the Romieu test were positive. Furthermore, the negative results obtained with the histochemical "precipitation" tests involving the formation of digitonides suggest the absence of "3-β-hydroxysterols or certain other steroids with the same configuration" (Cain, 1950) in the brown fat tissues of the bat. From these various tests applied to tissues collected throughout a year, esters of sterols (principally cholesterol esters) were therefore the only steroids that could be demonstrated histochemically in the present investigation.

To my knowledge, the histochemical tests involving the formation of digitonides previously had never been applied to brown fat tissues of any mammal. However, Baker, Ingle, and Li (1950) and Fawcett (1952) were unable to detect esters of sterols in the brown fat of rats by the histochemical "color" tests for steroids, and Baker, Ingle, and Li (1950) reported negative results with the brown fat of rats when the Liebermann-Burchardt test was applied to thick frozen sections extracted with warm chloroform. Using the biochemical approach, Clément (1947a) noted only faint traces of sterols in the brown fat of rats. On the contrary, Cramer (1920) observed that warm chloroform suspensions of dried brown fat of rats gave positive Liebermann-Burchardt reactions. Cramer also studied the brown fat of rabbits, guinea pigs, and mice, and he proposed that the brown fat tissue be called the "cholesterin gland" since it is especially rich in "cholesterin compounds." Thus, the findings on the steroid content of the brown fat of nonhibernating mammals are conflicting. Except for the relatively old observations of Cramer (1920), most results seem to indicate the absence of steroids from brown fat tissues of nonhibernating mammals. However, the only observation on the brown fat of hibernating mammals is in agreement with my histochemical and biochemical results.

Sweet and Hoskins (1940) found that the alcoholic extract of "hibernating glands" of woodchucks contained the equivalent of one International Unit of androgen per 50 gm. of tissue. In the present investigation, biochemical analyses reveal the presence of small amounts of total cholesterol in the interscapular brown fat of *Myotis lucifugus lucifugus*. The greatest concentration of total cholesterol is found in brown fat tissues collected during the summer months, and the concentrations are highest in August and lowest in December. Sweet and Hoskins (1940) believe that the high concentrations of androgen in woodchucks collected during the summer months "may reflect the part played by the hibernating gland in the sex cycle of the woodchuck." The same tentative explanation may be given for the higher levels of "total cholesterol" observed in bats collected during the summer months. But, like Sweet and Hoskins, I think that further investigation must be done before any functional significance can be ascribed to the presence of steroids in brown fat tissues of hibernating mammals such as the woodchuck and the bat.

To my knowledge, no quantitative biochemical analysis of the fatty acid content of brown fat has ever been reported. Carlier and Evans (1904) noted that the amount of stearic acid in this tissue increased toward the end of hibernation in the hedgehog. They suggest that oleic acid and the oleins may be oxidized more readily than the saturated acids and triglycerides, and that the unsaturated fatty acids may be the first to be called upon to meet the demands of the organism. The present investigation seems to indicate that the concentration of fatty acids is relatively high in the interscapular brown fat of *Myotis lucifugus lucifugus*, and that the quantitative variations of those fatty acids throughout the year parallel those of the total lipids. The present data are insufficient basis for speculation on the chemical nature of these fatty acids. This problem would prove an interesting topic for further research, and the contradictory results (Shattock, 1909; Coninx-Girardet, 1927; Fawcett, 1952) obtained on the degree of saturation of the fatty acids of brown adipose tissue of hedgehogs, marmots, and rats could thereby be at least partially elucidated.

The chemical nature of the phospholipids has also attracted the attention of workers interested in the lipid composition of brown fat. Cramer (1920), after examining frozen sections under crossed Nicol prisms, concludes that brown fat is rich in "cholesterin" compounds and "other lipoids in addition to true fat." This conclusion is supported by his own observations of doubly refractile material in the brown fat of rats. Cramer's results and conclusions are difficult to interpret, since the crystals he described may represent any lipids such as steroids, phospholipids, triglycerides, or fatty acids. Vignes (1913a) noted that the "hibernating mass" of rats is composed largely of a substance that is related to the lecithins and that is soluble in ether and chloroform, but insoluble in acetone. This compound may indeed be a lecithin, but galactolipids and other phospholipids also share some of these physical properties. Thus the findings of Vignes only vaguely suggest the presence of a phospholipid in brown fat, and a great deal more evidence is needed to prove that such a lecithin is present in brown adipose tissue. The same remarks hold true for Engelmann's observations (cited in Salmon, 1910) and Tonutti's (1936) on the occurrence of phospholipids in brown fat cells.

In contrast to these findings of large amounts of phospholipids in brown fat tissues, Carlier and Evans (1904) report the occurrence of only traces of lecithin in the "hibernating gland" of the hedgehog. These workers think that these traces are "no doubt derived partly from the medullary sheaths of the nerves within the organ, and partly from lecithin in mechanical combination with nucleoproteid in gland cells, etc." Unfortunately, no quantitative data are given by Carlier and Evans to substantiate their findings. Clément (1947a) merely states that phospholipids are found in such minute amounts that they cannot be determined quantitatively. My quantitative analyses on the phosphorus content of brown fat extracts also failed to reveal any amount of phosphorus detectable by biochemical means. However, the presence of phospholipids was implied by my histochemical tests, and Fawcett (1952) obtained similar histochemical results with the interscapular brown fat of rats.

These contradictions between the histochemical and the biochemical observations are believed to be due to the greater sensitivity and the specificity of the histochemical method. It seems that Baker's histochemical test for phospholipids is more sensitive than the known methods of microanalysis. This is partly inferred from Clément's (1947a) recent failure to detect phospholipids in brown fat tissues of rats, while Fawcett's (1952) histochemical reactions strongly suggest that phospholipids are present in the cytoplasm of the brown fat cells of the same animal species.

Baker's (1946) acid hematein test is specific for "lipins," but further evidence is required to prove definitely that it indicates only the phospholipids and does not give positive results with galactolipids. Experiments *in vitro* have shown that the acid hematein test gives negative reactions with galactolipids (Baker, 1947; Cain, 1950), but Lison (1953) rightly believes that studies on tissue sections are required before Baker's test can be finally designated as specific for phospholipids only.

That Baker's test is highly specific for phospholipids is inferred by its positive reaction with mitochondria (Baker, 1946). Lison (1953) maintains that "this direct histological proof of the existence of lipins in the chondriome must be considered as an important fact." A quantitative separation of liver mitochondria by differential centrifugation has shown that these cytoplasmic inclusions are made up of 25 to 30 per cent lipids. Of these lipid components, two thirds are phospholipids that occur in firm chemical association with the other components of the mitochondria (Claude, 1946 and 1949). This close association with the other components of the mitochondria may explain why the analyses of Clément (1947a) and my own determinations failed to reveal phospholipids by biochemical means.

According to Claude (1949), phospholipids "cannot be removed even by prolonged extraction with organic solvents... unless the complex is severely disrupted... by a variety of conditions such as heating at 55° C. for 30 minutes, ... treatment with chloroform, alcohol, or acetone, drying, and repeated freezing and thawing." Although, in my experiments, the brown fat extracts were treated with acetone and an alcohol-ether mixture, it is possible that these extractions at room temperature failed adequately to separate the phospholipids of the cells. With the interscapular brown fat of *Myotis lucifugus lucifugus*, Nelson and Tsuji (unpublished results) could not find any detectable amount of water in brown fat of this species. According to these workers, the low content of moisture could be explained by two facts; namely, the tissues had been stored for a too long period of time, and the drying technique employed lacked sensitivity. However, Nelson and Tsuji found small amounts of phospholipids in these dehydrated brown fat tissues after extraction with a boiling mixture of ethanol and ether in a Soxhlet apparatus. It seems, therefore, that my failure to detect any phospholipids by biochemical means may be due to the incomplete separation of the phospholipid-protein complexes of the brown fat cells.

The specific and highly sensitive reaction with Baker's histochemical tests indicates that the lipins are concentrated in cytoplasmic granules that seem to correspond to mitochondria. This observation in brown adipose tissues of *Myotis lucifugus lucifugus* corresponds to an analogous finding made by Fawcett (1952) in the brown fat of rats. Fawcett (1952) further noticed that the characteristic brown coloration of multilocular adipose tissue did not disappear completely after the blood was removed from the tissue. The color that persisted resembled that of the liver cleared of blood by perfusion. After washing out liver tissues, Bensley (1947) showed that the persisting coloration of these tissues was concentrated in the mitochondria and submicroscopic particles. The colored compounds appeared to be chemically related to the colored substances resulting from the auto-oxidation of phospholipids and certain unsaturated fatty acids, and the presence of such colored compounds in the mitochondria was interpreted as an indication that these organelles are the site of highly active oxidative processes related to the metabolism of fat. As Fawcett (1952) suggests, the presence in brown fat of numerous mitochondria rich in phospholipids "makes it easy to speculate that some of the color of this tissue may also be attributed to oxidation products of phospholipids." This suggestion is especially plausible if the formation of auto-oxidation products of phospholipids is associated with oxidative reactions in the metabolism of lipids.

SUMMARY AND CONCLUSIONS

The lipids of the interscapular brown adipose tissue of the female vespertilionid bat *Myotis lucifugus lucifugus* were studied by histochemical and microchemical methods during a full year.

(1) In order to refer the microchemical quantitative data to the fresh weights and the dry weights of brown fat tissue, the water content and the dry matter, or solids, were determined. From the end of August to the end of April, there is a gradual decrease in the total weight and in the water content of the interscapular brown fat. This gradual decrease in the moisture content of brown adipose tissue is possibly related to the depletion of water during hibernation.

(2) During the awakened period of *Myotis lucifugus lucifugus*, the water content of brown fat fluctuates. Thus, a high content of moisture is observed at the end of May and at the end of August; the amount of water in the interscapular brown fat is low at the beginning of August. These variations in the content of moisture of the interscapular lobes of the bat during its awakened period are believed to be associated with the more dynamic metabolic activity of *Myotis lucifugus lucifugus* during the summer months.

(3) The concentration of total lipids in the brown adipose tissues is comparatively high during the summer months. However, a decrease in these total lipids is observed at the end of August; that is, about one month before the onset of hibernation. This decline is followed by a sharp rise in the concentration of total lipids in the interscapular lobes of brown adipose tissue at the onset of hibernation which, in turn, is characterized by a drop in the amount of total lipids in the interscapular lobes of *Myotis lucifugus lucifugus*.

(4) It seems that the total fatty acids (free fatty acids, as well as esters of glycerol and cholesterol compounds) are metabolized actively during the summer months. The decreasing levels of fatty acids observed during hibernation may indicate that the interscapular brown fat acts as a reserve storage of total fatty acids that, together with the water, are depleted during the hibernating period. The slightly higher levels observed at the end of hibernation may represent a gradual storage of total fatty acids to meet the requirements of the approaching increased metabolic activity.

(5) The levels of total cholesterol are relatively high throughout the summer months. Low concentrations of total cholesterol are observed in brown fat tissues collected during the first months of hibernation, while higher levels are noted in tissues collected at the end of April.

(6) As with the total lipids and the total fatty acids, the total cholesterol seems to be more concentrated in brown fat at two times of the year: first, during the awakened period and, second, at the end of hibernation.

(7) It is possible that the variations in the levels of total cholesterol, total lipids, and total fatty acids reflect the general metabolic activities and, more specifically, the sexual activity of *Myotis lucifugus lucifugus*. Further investigation is needed before ascribing any definite functional significance to the fluctuations in the concentrations of the lipids and cholesterol of brown fat tissues of *Myotis lucifugus lucifugus*.

(8) Microchemical quantitative determinations of total phosphorus failed to reveal any detectable amount of phosphorus in the extracts from the interscapular lobes of *Myotis lucifugus lucifugus*. This failure to detect phosphorus in the tissues studied is thought to be due to the incomplete separation of the lipid-protein complexes of the brown fat cells.

(9) The anatomical distribution of the interscapular lobes of brown fat in *Myotis lucifugus lucifugus* is described for the first time. Variations in the extent and color of this tissue throughout the year are noted. The variations in color are thought to be due partly to changes in the vascularity of the tissue and partly to the relative amounts of white adipose tissue associated with the interscapular lobes of brown fat.

(10) The microscopical organization of the interscapular brown fat of *Myotis lucifugus lucifugus* is described, together with the variations in the diameter of the brown adipose cells at different periods of the year. A gradual increase in the size of the cell is noted as the hibernating period is approached, while a slight decrease in the diameter of the cell is observed during the hibernating period itself.

(11) The structural variations in the cytoplasmic droplets or "fat vacuoles" of the brown fat cell are described. In tissues collected in December, the whole extent of the interscapular lobe presents a uniform picture. In the lobes collected at the end of the hibernating period, a narrow "cortex" and a wide "medulla" can be recognized. The structural variations in the cytoplasmic droplets of brown fat cells in both regions of the interscapular lobe are described and discussed.

(12) From May to the end of August, two regions can also be distinguished in the interscapular lobes of *Myotis lucifugus lucifugus*. These regions and the structural variations in the sudanophilic droplets of the brown fat cytoplasm are described. The presence of very large lipid droplets in the cytoplasm of the cells during the summer months is believed to be a characteristic of brown fat tissues of *Myotis lucifugus lucifugus* during this period of the year. The differences between these large droplets of multilocular adipose tissue and the still larger droplets of unilocular adipose tissue are discussed.

(13) The qualitative variations in the histochemical composition of the cytoplasmic droplets of the brown fat cells were investigated throughout the year. It appears that these droplets are filled with substances of a purely lipid composition.

(14) During the summer months, the cytoplasmic droplets are especially rich in neutral lipids, principally esters of glycerol and esters of cholesterol and, possibly, lecithins and galactolipids. Traces of acidic lipids may also be present in these droplets during the summer months.

(15) During hibernation, there is an increase in acidic lipids (traces of oleic acid or other fatty acids, phospholipids, and chromolipids) in the cytoplasmic droplets of brown fat. A correlation of these histochemical observations and the microchemical quantitative data seem to indicate that the apparent increase in acidic lipids may be due to removal of neutral lipids from the interscapular lobes during the hibernating period.

(16) Esters of sterols (principally esters of cholesterol) are the only steroid compounds that could be detected by histochemical tests in the interscapular brown fat of *Myotis lucifugus lucifugus*. No variations in these sterol esters could be detected during a year's time by histochemical means. Throughout this period these steroid compounds were always found in the cytoplasmic droplets of the brown fat cell.

(17) The remainder of the cytoplasm of the brown fat cell seems to be filled with granules that apparently do not vary in composition, number, or size throughout the year.

(18) The cytochemical composition of these granules was demonstrated by Baker's acid hematein test, which is believed to be highly specific and sensitive. Comparisons between sections treated for the demonstration of mitochondria and sections stained by Baker's test suggest strongly that the cytoplasmic granules are mitochondria that are especially rich in phospholipids.

(19) The possible relation between the brown color of multilocular adipose tissue and the presence of oxidation products of phospholipids and of certain unsaturated fatty acids is discussed briefly.

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